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(54) Title: GFRα–X, A NOVEL GLIAL–DERIVED NEUROTROPHIC FACTOR RECEPTOR AND USES THEREFOR

(57) Abstract

The invention provides isolated nucleic acids molecules that encode new members of the GFRα family of protein, designated *GFRα–X* for GDNF Family Receptor Alpha–X nucleic acid molecules. This family of proteins bind neurotrophic factors and mediate signals involved in the regulation of neural cell functions. The invention also provides antisense nucleic acid molecules, expression vectors containing *GFRα–X* nucleic acid molecules, host cells into which the expression vectors have been introduced, and non–human transgenic animals in which a *GFRα–X* gene has been introduced or disrupted. The invention still further provides isolated GFRα–X polypeptides, fusion proteins, antigenic peptides, and anti–GFRα–X antibodies. Diagnostic, screening, and therapeutic methods utilizing compositions of the invention are also provided.

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GFRα-X, A NOVEL GLIAL-DERIVED NEUROTROPHIC FACTOR RECEPTOR AND USES THEREFOR

Background of the Invention

Two recently described growth factors, glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN), have been found to be approximately 20% similar to other TGF-β family members. This similarity is based primarily on the seven cysteine residues found in the same relative spacing across the entire TGF-β family of growth factors. Mature GDNF and mature NTN, however, have a high similarity (approximately 42%) to one another relative to the other TGF-β family members. Thus, these neurotrophic factors represent a subfamily of new growth factors within the TGF-β superfamily of growth factors and may be more closely related than their homology suggests. Due to their potent neurotrophic effects on a variety of neural cell types, moreover, GDNF and NTN comprise a subfamily of neurotrophic factors. As GDNF was cloned several years earlier than NTN (Lin et al. ((1993) *Science* 260(5111):1130-1132; Kotzbauer, P.T. et al. (1996) *Nature* 384:467-470), the effects of GDNF on various types of neural cells have been better characterized than the effects of NTN on neural cells.

GDNF was first characterized as promoting survival of cultured dopaminergic neurons of the substantia nigra (Lin et al. ((1993) *Science* 260(5111):1130-1132). GDNF was subsequently found to have potent effects on a wide range of additional neural populations. For example, GDNF was found to promote the survival of primary cultures of spinal motorneurons (Henderson, C.E. et al. (1994) *Science* 266:1062-1064) and mutated motorneurons *in vivo* (Li, L.X. et al. (1995) *PNAS* 92:9771-9775; Oppenheim, R. et al. (1995) *Nature* 373:344-346; Yan, Q. et al. (1995) *Nature* 373:341-344). Additionally, GDNF has been observed to have pronounced effects on cultures of dissociated neurons from various chick peripheral ganglia--sympathetic, sensory, and enteroceptive (Bujbello, A. et al. (1995) *Neuron* 15:821-828; Ebendal, T. et al. (1995) *Cell Growth & Diff.* 7:1081-1086;Trupp, M. et al. (1995) *J.Cell. Biol.* 130:137-148). GDNF has also been shown to promote the survival and morphologic differentiation of

primary cultures of Purkinje cells (Mount, H.T.J. et al. (1995) *PNAS* 92:9092-9096). Still other functions of GDNF include the ability to prevent degeneration and promote the phenotype of brain noradrenergic neurons *in vivo* (Arenas, E. et al. (1995) *Neuron* 15:1465-1473), to sustain axotomized basal forebrain cholinergic neurons *in vivo* (Williams, L.R. et al. (1996) *J. Pharmacol. Exp. Ther.* 277:1140-1151), and to inhibit kainic acid mediated seizures in rat (Martin, D. et al. (1995) *Brain Res.* 683:172-178). In addition to these effects in the adult central nervous systems, GDNF plays a critical role as a morphogen in the developing excretory and enteric nervous systems. This role is evident in the fact that mice defective in GDNF expression display complete renal agenesis and lack of enteric neurons (Moore, M.W. et al. (1996) *Nature* 382:76-79; Pichel, J.G. et al. (1996) *Nature* 382:73-76; Sanchez, M.P. et al. (1996) *Nature* 382:70-73).

NTN has been characterized as promoting survival of nodose ganglia sensory neurons, dorsal root ganglia sensory neurons, and superior cervical ganglia sympathetic neurons *in vitro* (Kotzbauer, P.T. et al. (1996) *Nature* 384:467-470). NTN's effect on other neural cell types has not yet been determined.

GDNF and NTN signal cells, e.g., neural cells and other cell types, in many instances, via a multicomponent receptor system formed by a glycosyl-phosphatidylinositol (GPI)-linked ligand binding subunit (the "α" subunit) and the tyrosine kinase receptor RET as a signaling ("β") subunit. Jing, S. et al. (1996) *Cell* 85:1113-1124; Treanor, J.J.S. et al. (1996) *Nature* 382:80-83. Binding of these neurotrophic factors to the α subunit promotes formation of a physical complex between the α and β subunits, thereby inducing tyrosine phosphorylation of the β subunit. Tyrosine phosphorylation of the β subunit results in transmission of the GDNF/NTN signal to the interior of the cell.

Several genes encoding α subunits of this GDNF/NTN receptor complex have been cloned and characterized. The first member of this receptor family, GDNF receptor-α (GDNFR-α), which has been renamed GFRα-1 for ***G***DNF ***F***amily ***R***eceptor ***A***lpha-***1*** by the GFRα Nomenclature Committee (GFRα Nomenclature Committee (1997) *Nature* 19:485) has been shown to bind to GDNF and to mediate binding and

activation of the RET receptor tyrosine kinase (Jing, S.Q. et al. (1996) *Cell* 85:1113-1124; Treanor, J.J.S. et al. (1996) *Nature* 382:80-83). The second member of the receptor family, alternatively named TrnR-2, NTNR-α, RETL2, and GDNFR-β, which has been renamed GFRα-2 by the GFRα Nomenclature Committee (GFRα Nomenclature Committee (1997) *Nature* 19:485), has been shown to bind NTN and to mediate activation of RET by both NTN and GDNF (Baloh, R.H. et al. (1997) *Neuron* 18:793-802; Bujbello, A. et al. (1997) *Nature* 387:721-724).

A third member of the receptor family, GRFα-3, has been described at recent scientific conferences.

Summary of the Invention

The invention is based on the discovery of nucleic acid molecules that encode a fourth member of the Glial Derived Neurotrophic Factor-Alpha Family of Receptors, hereinafter the *GFRα-X* cDNA, as well as the GFRα-X protein. The first member of the *GFRα-X* subfamily was identified, as described herein, in a positional cloning process in which the mouse mahogany locus was being sequenced to identify genes involved in obesity. Nucleic acid molecules encoding the GFRα-X proteins are referred to herein as *GFRα-X* nucleic acid molecules.

The GFRα-X proteins of the present invention bind to neurotrophic factors, such as GDNF and/or NTN, and mediate signals within cells expressing the GFRα-X protein. Typically, the GFRα-X protein transmits a signal to the interior of the cell by activation of the RET protein tyrosine kinase signalling pathway. Neurotrophic factors promote survival and function of neural cells of both the central and peripheral nervous systems. Thus, modulation of the activity of a molecule involved in transmitting a neurotrophic factor signal to a cell (e.g., GFRα-X) results in modulation of the neurotrophic factor initiated cell function. Consequently, modulation of GFRα-X function can be used to modulate neurotrophic factor action/activity and thereby treat disorders associated with such functions (or lack thereof).

In addition, GFRα proteins are expressed in a variety of cell lineages in the brain (for example Lateral septal neurons, Septohypothalamic neurons, paraventricular thalamic neurons (anterior), superchiasmatic neurons, anterior cortical amygdaloid neurons, piriform cortex, paracentral thalamic neurons, lateral habenular neurons, paraventricular hypothalamic neurons (PVN), amygdaloid nucleus area, arcuate neurons, and ventromedial hypothalamic neurons (VMH)) and during embryogenesis, including, for example, cells of the midbrain, motorneurons, cells of the enteric nervous system, embryonic smooth and striated muscles around the enteric nervous system in the esophagus, gut and stomach, developing nephrons and cells of the pancreatic primordium. Thus, modulators of GFRα-X can be used to modulate development of these tissues to thereby treat disorders associated with abnormal or aberrant development of these various tissues.

Accordingly, one aspect of the invention provides isolated nucleic acid molecules (e.g., cDNAs) comprising a nucleotide sequence encoding a GFRα-X protein or a fragment thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of GFRα-X-encoding nucleic acid (e.g., mRNA). In particularly preferred embodiments, the isolated nucleic acid molecule comprises a nucleic acid molecule which encodes the amino acid sequence of SEQ ID NO:2, such as the nucleotide sequence of SEQ ID NO:1. In other particularly preferred embodiments, the isolated nucleic acid molecule of the invention comprises a nucleotide sequence which hybridizes to or is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence shown in SEQ ID NO:1.

In another embodiment, the isolated nucleic acid molecule encodes a protein or portion thereof wherein the protein or portion thereof includes an amino acid sequence which is sufficiently homologous to a protein comprising an amino acid sequence of SEQ ID NO:2, such that the protein or portion thereof maintains a GFRα-X activity. Preferably, the protein or portion thereof encoded by the nucleic acid molecule maintains the ability to bind to a neurotrophic factor and modulate a cellular response. In one embodiment, the protein encoded by the nucleic acid molecule is at least about

30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the amino acid sequence of SEQ ID NO:2. In another preferred embodiment, the protein is a full length protein which is substantially homologous to the entire amino acid sequence of SEQ ID NO:2, such the naturally occurring full length protein, and all allelic variants and splice variants of human and murine GFRα-X.

In another embodiment, the isolated nucleic acid molecule is at least 15 nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1. Preferably, the isolated nucleic acid molecule corresponds to a naturally occurring nucleic acid molecule. More preferably, the isolated nucleic acid encodes naturally-occurring alleles and splice variants of human GFRα-X. Moreover, given the disclosure herein of a GFRα-X-encoding cDNA sequence (e.g., SEQ ID NO:1), antisense nucleic acid molecules (i.e., molecules which are complementary to the coding strand of the GFRα-X cDNA sequence) are also provided by the invention.

Another aspect of the invention pertains to vectors, e.g., recombinant expression vectors, containing the nucleic acid molecules of the invention and host cells into which such vectors have been introduced. In one embodiment, such a host cell is used to produce GFRα-X protein by culturing the host cell in a suitable medium. If desired, the GFRα-X protein can then be isolated from the host cell.

Yet another aspect of the invention pertains to transgenic non-human animals in which a *GFRα-X* gene has been introduced or altered. In one embodiment, the genome of the non-human animal has been altered by introduction of a nucleic acid molecule of the invention encoding GFRα-X as a transgene. In another embodiment, an endogenous *GFRα-X* gene within the genome of the non-human animal has been altered, e.g., functionally disrupted, by homologous recombination.

Still another aspect of the invention pertains to an isolated GFRα-X protein or a portion, e.g., a biologically active portion, thereof. In a preferred embodiment, the isolated GFRα-X protein or portion thereof can bind a neurotrophic factor and stimulate a response in a neurotrophic factor responsive cell.

The invention also provides an isolated preparation of a GFRα-X protein. In preferred embodiments, the GFRα-X protein comprises the amino acid sequence of SEQ ID NO:2. In another preferred embodiment, the invention pertains to an isolated full length protein which is substantially homologous to the entire amino acid sequence of SEQ ID NO:2 (containing additional 5' sequence). In yet another embodiment, the protein is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the entire amino acid sequence of SEQ ID NO:2. In other embodiments, the isolated GFRα-X protein comprises an amino acid sequence which is at least about 60-70% or more homologous to the amino acid sequence of SEQ ID NO:2 and has one or more of the following activities: 1) it can interact with (e.g., bind to) a neurotrophic factor, e.g., GDNF and/or NTN; 2) it can interact with (e.g., bind to) a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; 3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and 4) it can bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell, e.g., a neural cell, a cell of the developing digestive tract, or a cell of its associated nervous system innervation, to, for example, beneficially affect the cell. Alternatively, the isolated GFRα-X protein can comprise an amino acid sequence which is encoded by a nucleotide sequence which hybridizes, e.g., hybridizes under stringent conditions, or is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence of SEQ ID NO:1. It is also preferred that the preferred forms of GFRα-X also have one or more of the GFRα-X activities described herein.

The GFRα-X protein (or polypeptide) or a biologically active portion thereof can be operatively linked to a non-GFRα-X polypeptide to form a fusion protein.

The GFRα-X protein of the invention, or portions or fragments thereof, can be used to prepare anti-GFRα-X antibodies. Accordingly, the invention also provides an antigenic peptide of GFRα-X which comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of GFRα-X such that an antibody raised against the peptide forms a specific immune complex with GFRα-X. Preferably, the antigenic peptide comprises at least 10 amino acid residues,

more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues and has a high antigenicity index as shown in Figure 2. The invention further provides an antibody that specifically binds GFRα-X. In one embodiment, the antibody is monoclonal. In another embodiment, the antibody is coupled to a detectable substance. In yet another embodiment, the antibody is incorporated into a pharmaceutical composition comprising the antibody and a pharmaceutically acceptable carrier.

Another aspect of the invention pertains to methods for modulating a GFRα-X mediated cell activity, e.g., function, proliferation or differentiation. Such methods include contacting the cell with an agent which modulates a GFRα-X protein activity or *GFRα-X* nucleic acid expression such that a cell associated activity is altered relative to a cell associated activity (e.g., the same cell associated activity) of the cell in the absence of the agent. In a preferred embodiment, the cell is capable of responding to a neurotrophic factor through a signaling pathway involving a GFRα-X protein. The agent which modulates GFRα-X activity can be an agonist agent, an agent which stimulates GFRα-X protein activity or *GFRα-X* nucleic acid expression; or an antagonist agent, an agent which inhibits GFRα-X protein activity or *GFRα-X* nucleic acid expression. Examples of agents which stimulate GFRα-X protein activity or *GFRα-X* nucleic acid expression include small molecules and nucleic acids encoding GFRα-X that have been introduced into the cell. Examples of agents which inhibit GFRα-X activity or expression include small molecules, antisense *GFRα-X* nucleic acid molecules, and antibodies that specifically bind to GFRα-X. In a preferred embodiment, the cell is present within a subject and the agent is administered to the subject.

The present invention also pertains to methods for treating subjects having disorders mediated by abnormal GFRα-X activity/expression. For example, the invention pertains to methods for treating a subject having a disorder characterized by aberrant GFRα-X protein activity or nucleic acid expression such as a neurological disorder, e.g., a central nervous system disorder, e.g., Parkinson's disease, or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development. These methods

include administering to the subject a GFRα-X modulator (e.g., a small molecule) such that treatment of the subject occurs.

In another embodiment, the invention pertains to methods for treating a subject having a neurological disorder, e.g., a central nervous system disorder, e.g., Parkinson's disease, or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development, comprising administering to the subject a GFRα-X modulator such that treatment occurs.

In other embodiments, the invention pertains to methods for treating a subject having a neurological disorder, e.g., a central nervous system disorder, e.g., Parkinson's disease or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development, comprising administering to the subject a GFRα-X protein or portion thereof such that treatment occurs. Neurological disorders and disorders associated with abnormal or aberrant cell, e.g., neural cell, development can also be treated according to the invention by administering to the subject having the disorder a nucleic acid encoding a GFRα-X protein or portion thereof such that treatment occurs.

The invention also pertains to methods for detecting genetic mutations in a *GFR α-X* gene, thereby determining if a subject with the mutated gene is at risk for (or is predisposed to have) a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity, e.g., a central nervous system disorder, e.g., Parkinson's disease or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic mutation characterized by an alteration affecting the integrity of a gene encoding a GFRα-X protein, or the misexpression of the *GFRα-X* gene.

Another aspect of the invention pertains to methods for detecting the presence of GFRα-X, or fragment thereof, in a biological sample. In a preferred embodiment, the methods involve contacting a biological sample (e.g., a neural cell sample) with a compound or an agent capable of detecting GFRα-X protein or GFRα-X encoding mRNA such that the presence of GFRα-X is detected in the biological sample. The compound or agent can be, for example, a labeled or labelable nucleic acid probe

capable of hybridizing to GFRα-X encoding mRNA or a labeled or labelable antibody capable of binding to GFRα-X protein. The invention further provides methods for diagnosis of a subject with, for example, a central nervous system disorder, e.g., Parkinson's disease, or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development, based on detection of GFRα-X protein or mRNA. In one embodiment, the method involves contacting a cell, tissue, or fluid sample (e.g., a neural cell sample) from the subject with an agent capable of detecting GFRα-X protein or mRNA, determining the amount of GFRα-X protein or mRNA expressed in the sample, comparing the amount of GFRα-X protein or mRNA expressed in the sample to a control sample and forming a diagnosis based on the amount of GFRα-X protein or mRNA expressed in the sample as compared to the control sample. Preferably, the sample is a neural cell sample. Kits for detecting GFRα-X, or fragments thereof, in a biological sample are also within the scope of the invention.

Still another aspect of the invention pertains to methods, e.g., screening assays, for identifying a compound for treating a disorder characterized by aberrant *GFRα-X* nucleic acid expression or protein activity, e.g., a central nervous system disorder, e.g., Parkinson's disease or a disorder associated with abnormal or aberrant cell, i.e., neural cell, development. These methods typically include assaying the ability of the compound or agent to modulate the expression of the *GFRα-X* gene or the activity of the GFRα-X protein thereby identifying a compound for treating a disorder characterized by aberrant *GFRα-X* nucleic acid expression or protein activity. In a preferred embodiment, the method involves contacting a biological sample obtained from a subject having the disorder with the compound or agent, determining the amount of GFR α-X protein expressed and/or measuring the activity of the GFRα-X protein in the biological sample, comparing the amount of GFRα-X protein expressed in the biological sample and/or the measurable GFRα-X biological activity in the cell to that of a control sample. An alteration in the amount of GFRα-X protein expression or GFRα-X activity in the cell exposed to the compound or agent in comparison to the control is indicative of a modulation of GFRα-X expression and/or GFRα-X activity.

The invention also pertains to methods for identifying a compound or agent which interacts with (e.g., binds to) a GFRα-X protein. These methods can include the steps of contacting the GFRα-X protein, a fragment thereof, or a cell expressing GFRα-X, with the compound or agent under conditions which allow binding of the compound to the GFRα-X protein to form a complex and detecting the formation of a complex of the GFRα-X protein and the compound in which the ability of the compound to bind to the GFRα-X protein is indicated by the presence of the compound in the complex.

The invention further pertains to methods for identifying a compound or agent which modulates, e.g., stimulates or inhibits, the interaction of the GFRα-X protein with a target molecule, e.g., GDNF, NTN, a complex of GDNF and NTN, or the tyrosine kinase receptor RET. In these methods, the GFRα-X protein is contacted, in the presence of the compound or agent, with the target molecule under conditions which allow binding of the target molecule to the GFRα-X protein to form a complex. An alteration, e.g., an increase or decrease, in complex formation between the GFRα-X protein and the target molecule as compared to the amount of complex formed in the absence of the compound or agent is indicative of the ability of the compound or agent to modulate the interaction of the GFRα-X protein with a target molecule.

Brief Description of the Drawing

Figure 1 depicts the mouse GFRα-X nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence.

Figure 2 depicts a structural analysis of the mouse GFRα-X protein.

Figure 3 provides an alignment of the amino acid sequence of members of the GRFα family of protein.

Detailed Description of the Invention

The present invention is based on the discovery of novel molecules, referred to herein as *GFRα-X* nucleic acid molecules and GFRα-X proteins, which function in neurotrophic factor signaling pathways. As used herein, "a neurotrophic factor" refers to a protein that modulates a biological activity of a cell, particularly a neuronal cell,

through a neurotrophic factor signaling pathway, such as the RET signaling pathway. Examples of biological activities include, but are not limited to, neural cell survival and/or neural cell function. Examples of such neurotrophic factors include GDNF and NTN.

As used herein, "RET signaling pathway" includes a cell, e.g., neural cell, signaling pathway which involves the tyrosine kinase receptor RET. An example of such a pathway includes the GDNF or NTN (neurotrophic factor) signaling pathway.

As used herein, "a neurotrophic factor responsive cell" includes a cell which has a biological activity that can be modulated (e.g., stimulated or inhibited) by a neurotrophic factor. Examples of such functions include mobilization of intracellular molecules which participate in a signal transduction pathway, production or secretion of molecules, alteration in the structure of a cellular component, cell proliferation, cell migration, cell differentiation, and cell survival. Cells responsive to neurotrophic factors preferably express a neurotrophic factor receptor, e.g., a GFRα receptor, such as GFRα-X, and/or a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET. Examples of neurotrophic factor responsive cells include neural cells, e.g., cells of the central nervous system and peripheral nervous system cells (e.g., sympathetic and parasympathetic neurons), cells of the enteric nervous system, embryonic smooth and striated muscles around the enteric nervous system in the esophagus, gut and stomach, developing nephrons, and cells of the pancreatic primordium.

Depending on the type of cell, the response elicited by neurotrophic factors is different. For example, in neural cells, neurotrophic factors regulate neural survival and neural function. Abnormal or aberrant activity of proteins involved in the neurotrophic signaling pathway can lead to a variety of neurological disorders, e.g., central nervous system disorders. For example, abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the thalamus (e.g., the reticular thalamic nucleus, the zona certa, the anteromedial and dorsal thalamic nucleus, the lateral habenular nucleus, and the medial habenular nucleus) can lead to sensory disorders. Sensory disorders are disorders which detrimentally affect normal sensory function. Examples of such sensory disorders include Dejerine-Roussy syndrome, contralateral

anesthesia, and dense hypesthesia. Abnormal or aberrant activity in cells of the peripheral or enteric nervous system can lead to eating disorders.

Abnormal or aberrant activity of a GFRα-X (or abnormal or aberrant nucleic acid expression of the nucleic acid encoding the protein)in a neurotrophic signaling factor pathway in the midbrain or mesencephalon (e.g., the substantia nigra compacta and scattered cells of SN reticulata, the ventral segmental area, the interpenduncular nucleus, the supramammilary nucleus, the red nucleus, and the dorsal raphe nucleus) can lead to motor disorders. Motor disorders are disorders which detrimentally affect normal motor functions. Examples of such motor disorders include ataxia, facial infarction, tremors, tics, athetosis, amyotrophic lateral sclerosis (ALS), and Parkinson's disease.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the cerebellum (e.g., the Purkinje layer, the molecular layer, and the deep cerebellar nuclei) can also lead to motor disorders. Examples of such motor disorders include loss of equilibrium and multiple sclerosis.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the pons (e.g., the pontine reticular nucleus, the pontine nucleus, the motor trigeminal nucleus, the inferior olive nuclei, the locus coeruleus, the dorsal cochlear nucleus, the facial nucleus, the vestibular nucleus, and the hypoglossal nucleus) can lead to motor disorders. Examples of such motor disorders include facial palsy, and limb ataxia.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the olfactory system (e.g., the olfactory tubercule, the internal granular layer of olfactory bulb, the external plexiform layer of olfactory bulb, the glomerular layer, and the olfactory nerve layer) can lead to sensory disorders. Sensory disorders are disorders which detrimentally affect normal sensory function. An example of such a sensory disorder includes the loss of olfaction functionality.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor in a signaling pathway in the neocortex (also known as the neopallium or isocortex) (e.g., the hippocampus) can lead to cognitive disorders. Cognitive disorders are disorders which

detrimentally affect normal cognitive functions. An example of such a cognitive disorder is Alzheimer's disease.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the amygdala can lead to motor disorders. Examples of such motor disorders include athetosis, dystoia, and tremors.

In addition, neurotrophic factors such as GDNF and NTN and the interaction with GFRs also promote proper development of a variety of cell types. For example, neurotrophic factor/receptor interactions promote development and function of certain peripheral organs and cells of their associated nervous system innervation. Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in these peripheral organs (e.g., kidneys, testis, intestine, stomach, heart, lung and skin) can lead to disorders associated with cellular development of cells of these organs. An example of a disorder associated with development of the enteric nervous system is Hirschsprung's disease. Examples of disorders associated with development of the kidneys include kidney dysfunction, renal agenesis, and severe dysgenesis.

A murine *GFRα-X* nucleic acid molecule was identified from a positional cloning process in which the mouse mahogany locus was being sequenced to identify genes involved in obesity (described in detail in Example 1). During sequencing of a larger genomic region, an open reading frame was identified that encoded a protein that showed sequence homology to GFRα-1. Probes were generated based on portions of the genomic sequence and cDNA libraries were screened. Nucleotide sequences were determined and assembled and various methods such as RACE and genomic sequence analysis were used to extend the 5' sequence. The nucleotide sequence of the isolated mouse *GFRα-X* cDNA and the predicted amino acid sequence of the mouse GFRα-X protein are shown in Figure 1 and in SEQ ID NOs:1 and 2, respectively. A plasmid containing the nucleotide sequence encoding mouse GFRα-X was deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, on _____ and assigned Accession Number __. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was

made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

The murine *GFRα-X* nucleic acid molecule is approximately 1019 nucleotides in length, and encodes a protein that is approximately 339 amino acid residues in length. This clone is likely to be missing several nucleotides (and amino acids) that are present at the 5' end of the naturally occurring cDNA. The GFRα-X protein is expressed at least in brain cells, particularly in Lateral septal neurons, Septohypothalamic neurons, paraventricular thalamic neurons (anterior), superchiasmatic neurons, anterior cortical amygdaloid neurons, piriform cortex, paracentral thalamic neurons, lateral habenular neurons, paraventricular hypothalamic neurons (PVN), amygdaloid nucleus area, arcuate neurons, and ventromedial hypothalamic neurons (VMH).

Various aspects of the invention are described in further detail in the following subsections:

I. Isolated Nucleic Acid Molecules

One aspect of the invention provides isolated nucleic acid molecules that encode GFRα-X proteins, particularly human or murine orthologues, biologically active portions thereof, as well as nucleic acid fragments sufficient for use as hybridization probes to identify GFRα-X-encoding nucleic acid molecules (e.g., GFRα-X encoding mRNA). As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule" includes nucleic acid molecules which are separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and

3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated *GFRα-X* nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived (e.g., a neural cell). Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, a homologue or orthologue or human or murine *GFRα-X* cDNA can be isolated from a cDNA library, such as a brain library, using all or portion of SEQ ID NO:1 as a hybridization probe and standard hybridization techniques (e.g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1 can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO:1. For example, mRNA can be isolated from neural cells (e.g., by the guanidinium-thiocyanate extraction procedure of Chirgwin et al. (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (e.g., Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon the nucleotide sequences shown in SEQ ID NO:1. A nucleic acid of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

Furthermore, oligonucleotides corresponding to a *GFRα-X* nucleotide sequence can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1. The sequence of SEQ ID NO:1 corresponds to the mouse *GFRα-X* cDNA.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1 or a portion of this nucleotide sequence. For example, a nucleic acid molecule which is complementary to the nucleotide sequence shown in SEQ ID NO:1 is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1 such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1, thereby forming a stable duplex.

In still another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence which is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence shown in SEQ ID NO:1. In an additional preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence which hybridizes, e.g., hybridizes under stringent conditions, to the nucleotide sequence shown in SEQ ID NO:1 or a portion of either of these nucleotide sequences. Preferably such nucleic acid molecules encode naturally occurring allelic variants of the mouse *GFRα-X* nucleic acid molecules disclosed herein or non-mouse orthologues, such as human GFRα-X.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the coding region of SEQ ID NO:1, for example a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of GFRα-X such as a ligand binding domain or signaling partner binding site of GFRα-X. The nucleotide sequence determined from the cloning of the *GFRα-X* gene from a mouse allows for the generation of probes and primers designed for use in identifying and/or cloning *GFRα-X* homologues in other cell types, e.g., from other tissues, as well as *GFRα-X* orthologues from other mammals such as humans. The probe/primer typically comprises

substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 40, 50 or 75 consecutive nucleotides of SEQ ID NO:1 sense, an anti-sense sequence of SEQ ID NO:1, or naturally occurring mutants thereof. Primers based on the nucleotide sequence in SEQ ID NO:1 can be used in PCR reactions to clone GFRα-X homologues. Probes based on the GFRα-X nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto, e.g., the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a GFRα-X protein, such as by measuring a level of a GFRα-X-encoding nucleic acid in a sample of cells from a subject, e.g., detecting GFRα-X encoding mRNA levels or determining whether a genomic *GFRα-X* gene has been mutated or deleted.

In one embodiment, the nucleic acid molecule of the invention encodes a protein or portion thereof which includes an amino acid sequence which is sufficiently homologous to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof maintains one or more of the activities possessed by GFRα-X. Examples of such homologous proteins include, but are not limited to, allelic variants of SEQ ID NO:2 and non-mouse orthologues (such as human GFRα-X) of SEQ ID NO:2. As used herein, the language "sufficiently homologous" refers to proteins or portions thereof which have amino acid sequences which include a minimum number of identical or equivalent (e.g., an amino acid residue which has a similar side chain as an amino acid residue in SEQ ID NO:2) amino acid residues to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof is able to bind a neurotrophic and modulate a response in a neurotrophic factor responsive cell. In another embodiment, the protein is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the entire amino acid sequence of SEQ ID NO:2.

Portions of proteins encoded by the *GFRα-X* nucleic acid molecule of the invention preferably possess one or more activities of the GFRα-X protein. As used herein, the term "biologically active portion of GFRα-X" is intended to include a portion, e.g., a domain/motif, of GFRα-X that has one or more of the following activities: 1) it can interact with (e.g., bind to) a neurotrophic factor, e.g., GDNF and/or NTN; 2) it can interact with (e.g., bind to) a tyrosine kinase receptor or other signaling partner, e.g., the tyrosine kinase receptor RET; 3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and 4) it can bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell, e.g., a neural cell, a cell of the developing digestive tract, or a cell of its associated nervous system innervation, to, for example, beneficially affect the cell. Direct binding assays as described herein, can be performed to determine the ability of a GFRα-X protein or biologically active portion thereof to interact with (e.g., bind to) a neurotrophic factor (e.g., GDNF and/or NTN or a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET).

The ability of GFRα-X proteins of the present invention to interact with a neurotrophic factor can be determined using the following assay. Plasmids including a nucleic acid molecule which encodes a fragment of the mouse GFRα-X protein either alone or as a chimeric fusion protein with, for example, an Ig constant region can be generated, as described in Sanicola et al. (1991) *Proc. Natl. Acad. Sci.* 94:6238-6243, by ligating a DNA fragment encoding the GFRα-X fragment to suitable vector sequences. The plasmids can be transfected into 293-EBNA cells and stable lines obtained by using hygromycin selection. The GFRα-X fragment or fusion proteins can be purified and then exposed to rhGDNF (Promega, Madison, WI). Complexes of GFRα-X and rhGDNF can then be identified.

The ability of GFRα-X protein or a biologically active portion thereof to interact with (e.g., bind to) a tyrosine kinase receptor (e.g., the tyrosine kinase receptor RET) can be determined using an assay similar to the assay described above for determining the ability of a GFRα-X protein or biologically active portion thereof to interact with (e.g., bind to) a neurotrophic factor (e.g., GDNF and/or NTN). In particular, the mouse GFRα

-X protein as described above can be exposed to proteins known to complex with members of the GFRα-X family of receptors. Complexes can be identified and detected using art known methods.

The ability of a fragment of a GFRα-X protein of the present invention to modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET, can be determined using the following assay. As described in Treanor et al. (1996) *Nature* 382:80-83, the human neuroblastoma SK-N-SH and the mouse neuroblastoma Neuro-2a, cell lines that express endogenous c-*ret*, can be exposed to GDNF alone or to GDNF in combination with a soluble fragment of GFRα-X for 5 minutes, and the level of RET tyrosine phosphorylation can be determined. To determine whether induction of RET tyrosine phosphorylation is dependent on the presence of the GFRα-X fragment, Neuro-2a and SK-N-SH cells can be treated with PIPLC, and the response of RET to GDNF can be examined. A change in tyrosine kinase RET phosphorylation in the cell lines treated with GDNF in combination with soluble GFRα-X compared to cell lines treated with GDNF alone indicates that the GFR α-X protein is capable of modulating the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET.

In one embodiment, the biologically active portion of GFRα-X comprises the N-terminal domain of the GFRα-X protein. Figure 2 provides a structural analysis of the mouse GFRα-X protein. Additional domains can e identified by analyzing conserved residue in the GFRα family of proteins (Figure 3). Additional nucleic acid fragments encoding biologically active portions of GFRα-X can be prepared by isolating a portion of SEQ ID NO:1, expressing the encoded portion of GFRα-X protein or peptide (e.g., by recombinant expression *in vitro*) and assessing the activity of the encoded portion of GFRα-X protein or peptide.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1 (and portions thereof) due to degeneracy of the genetic code and thus encode the same GFRα-X protein as that encoded by the nucleotide sequence shown in SEQ ID NO:1. In such an embodiment, an isolated

nucleic acid molecule of the invention has a nucleotide sequence encoding a protein comprising an amino acid sequence shown in SEQ ID NO:2.

In addition to the GFRα-X nucleotide sequence shown in SEQ ID NO:1, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of GFRα-X may exist within a population. Such genetic polymorphism in the *GFRα-X* gene may exist among individuals within a population due to natural allelic variation producing both active variants and inactive variants. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a GFRα-X protein, preferably a mammalian GFRα-X protein. Such active natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the *GFRα-X* gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in GFRα-X that are the result of natural allelic variation are intended to be within the scope of the invention. Moreover, nucleic acid molecules encoding GFRα-X proteins from other species, and thus which have a nucleotide sequence which differs from the mouse sequence of SEQ ID NO:1, are intended to be within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and human homologues of the mouse *GFRα-X* cDNA of the invention can be isolated based on their homology to the mouse *GFRα-X* nucleic acid disclosed herein using the mouse cDNA, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 15 nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1. In other embodiments, the nucleic acid is at least 30, 50, 100, 250 or 500 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least about 65%, more preferably at least about 70%, and even more preferably at least about 75% or more homologous to each other typically remain hybridized to each other. Such stringent

conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1 corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g., encodes a natural protein) and is encoded by a common genetic locus. In one embodiment, the nucleic acid encodes a natural human GFRα-X.

In addition to naturally-occurring allelic variants of the GFRα-X sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by directed or random mutation into the nucleotide sequence of SEQ ID NO:1, thereby leading to changes in the amino acid sequence of the encoded GFRα-X protein, without altering the functional ability of the GFRα-X protein. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence of SEQ ID NO:1. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of GFRα-X (e.g., the sequence of SEQ ID NO:2) without altering the activity of GFRα-X, whereas an "essential" amino acid residue is required for GFRα-X activity. For example, conserved amino acid residues, e.g., hydrophobic amino acids, in the N-terminal domain of GFRα-X are most likely important for binding to a neurotrophic factor and are thus essential residues of GFRα-X. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved in the N-terminal hydrophobic domain) may not be essential for activity and thus are likely to be amenable to alteration without altering GFRα-X activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding GFRα-X proteins that contain changes in amino acid residues that are not essential for GFRα-X activity. Such GFRα-X proteins differ in amino acid sequence

from SEQ ID NO:2 yet retain at least one of the GFRα-X activities described herein. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:2 and is capable of binding a neurotrophic factor and modulating a response in a neurotrophic factor responsive cell. Preferably, the protein encoded by the nucleic acid molecule is at least about 70% homologous to SEQ ID NO:2, more preferably at least about 80-85% homologous to SEQ ID NO:2, even more preferably at least about 90% homologous to SEQ ID NO:2, and most preferably at least about 95-99% homologous to SEQ ID NO:2.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (e.g., when aligning a second sequence to the GFRα-X amino acid sequence of SEQ ID NO:2 having 177 amino acid residues, at least 80, preferably at least 100, more preferably at least 120, even more preferably at least 140, and even more preferably at least 150, 160 or 170 amino acid residues are aligned). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to GFRα-X nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to GFRα-X protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

An isolated nucleic acid molecule encoding a GFRα-X protein homologous to the protein of SEQ ID NO:2 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO:1 by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in GFRα-X is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a GFRα-X coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for a GFRα-X activity described herein to identify mutants that retain GFRα-X activity. Following mutagenesis of SEQ ID NO:1, the encoded protein can be expressed recombinantly (e.g., as described in Examples 4 and 5) and the activity of the protein can be determined using, for example, assays described herein.

In addition to the nucleic acid molecules encoding GFRα-X proteins described above, another aspect of the invention pertains to isolated nucleic acid molecules which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or

complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire GFRα-X coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding GFRα-X. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. Given the coding strand sequences encoding GFRα-X disclosed herein (e.g., SEQ ID NO:1), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of GFRα-X encoding mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of GFRα-X encoding mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of GFRα-X encoding mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-

methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a GFRα-X protein to thereby inhibit expression of the protein, e.g., by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementary to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of an antisense nucleic acid molecule of the invention includes direct injection at a tissue site. Alternatively, an antisense nucleic acid molecule can be modified to target selected cells and then administered systemically. For example, for systemic administration, an antisense molecule can be modified such that it specifically binds to a receptor or an antigen expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecule to a peptide or an antibody which binds to a cell surface receptor or antigen. The antisense nucleic acid molecule can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier et al. (1987) *Nucleic Acids.*

Res. 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al. (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analog (Inoue et al. (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (e.g., hammerhead ribozymes (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave GFRα-X encoding mRNA transcripts to thereby inhibit translation of GFRα-X encoding mRNA. A ribozyme having specificity for a GFRα-X-encoding nucleic acid can be designed based upon the nucleotide sequence of a *GFRα-X* cDNA disclosed herein (i.e., SEQ ID NO:1). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a GFRα-X-encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071 and Cech et al. U.S. Patent No. 5,116,742. Alternatively, GFRα-X encoding mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

Alternatively, *GFRα-X* gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the *GFRα-X* gene (e.g., the GFRα-X promoter and/or enhancers) to form triple helical structures that prevent transcription of the *GFRα-X* gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. et al. (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioassays* 14(12):807-15.

II. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding GFRα-X (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid",

which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art

that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., GFRα-X proteins, mutant forms of GFRα-X, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of GFRα-X in prokaryotic or eukaryotic cells. For example, GFRα-X can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. In one embodiment, the coding sequence of the GFRα-X is cloned into a pGEX

expression vector to create a vector encoding a fusion protein comprising, from the N-terminus to the C-terminus, GST-thrombin cleavage site-GFRα-X. The fusion protein can be purified by affinity chromatography using glutathione-agarose resin. Recombinant GFRα-X unfused to GST can be recovered by cleavage of the fusion protein with thrombin.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the GFRα-X expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, GFRα-X can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to GFRα-X encoding mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, GFRα-X protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized

techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding GFRα-X or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (i.e., express) GFRα-X protein. Accordingly, the invention further provides methods for producing GFRα-X protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding GFRα-X has been introduced) in a suitable medium until GFRα-X is produced. In another embodiment, the method further comprises isolating GFRα-X from the medium or the host cell.

The host cells of the invention can also be used to produce non-human transgenic animals. The non-human transgenic animals can be used in screening assays designed to identify agents or compounds, e.g., drugs, pharmaceuticals, etc., which are capable of ameliorating detrimental symptoms of selected disorders such as neurological disorders and morphological disorders. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which GFRα-X-coding

sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous GFRα-X sequences have been introduced into their genome or homologous recombinant animals in which endogenous GFRα-X sequences have been altered. Such animals are useful for studying the function and/or activity of GFRα-X and for identifying and/or evaluating modulators of GFRα-X activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous *GFRα-X* gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing GFRα-X-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The human homologue of the mouse *GFRα-X* nucleic acid molecule of SEQ ID NO:1 can be isolated based on hybridization to the mouse *GFRα-X* cDNA (described further in subsection I above) and used as a transgene, e.g., introduced as a transgene into the genome of a non-human animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the GFRα-X transgene to direct expression of GFRα-X protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both

by Leder et al., U.S. Patent No. 4,873,191 by Wagner et al. and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the GFRα-X transgene in its genome and/or expression of GFRα-X encoding mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding GFRα-X can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a *GFRα-X* gene into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the *GFRα-X* gene. The *GFRα-X* gene can be a human gene (e.g., from a human genomic clone isolated from a human genomic library screened with the cDNA of SEQ ID NO:1), but more preferably, is a non-human homologue of a human *GFRα-X* gene. For example, the mouse *GFRα-X* gene can be used to construct a homologous recombination vector suitable for altering an endogenous *GFRα-X* gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous *GFRα-X* gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous *GFRα-X* gene is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous GFRα-X protein). In the homologous recombination vector, the altered portion of the *GFRα-X* gene is flanked at its 5' and 3' ends by additional nucleic acid of the *GFRα-X* gene to allow for homologous recombination to occur between the exogenous *GFRα-X* gene carried by the vector and an endogenous *GFRα-X* gene in an embryonic stem cell. The additional flanking *GFRα-X* nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas, K.R. and

Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced *GFRα-X* gene has homologously recombined with the endogenous *GFRα-X* gene are selected (see e.g., Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec et al.; WO 91/01140 by Smithies et al.; WO 92/0968 by Zijlstra et al.; and WO 93/04169 by Berns et al.

In another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, e.g., Lakso et al. (1992) *PNAS* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. et al. (1997) *Nature* 385:810-813 and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit

the growth cycle and enter G_0 phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

III. Isolated GFRα-X Proteins and Anti-GFRα-X Antibodies

Another aspect of the invention pertains to isolated GFRα-X proteins, and biologically active portions thereof, as well as peptide fragments suitable for use as immunogens to raise anti-GFRα-X antibodies. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of GFRα-X protein in which the protein is separated from cellular components of the cells in which it is naturally or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of GFRα-X protein having less than about 30% (by dry weight) of non-GFRα-X protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-GFRα-X protein, still more preferably less than about 10% of non-GFRα-X protein, and most preferably less than about 5% non-GFRα-X protein. When the GFRα -X protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation. The language "substantially free of chemical precursors or other chemicals" includes preparations of GFRα-X protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of GFRα-X protein having less than about 30% (by dry weight) of chemical precursors or non-GFRα

-X chemicals, more preferably less than about 20% chemical precursors or non-GFRα-X chemicals, still more preferably less than about 10% chemical precursors or non-GFRα-X chemicals, and most preferably less than about 5% chemical precursors or non-GFRα-X chemicals. In preferred embodiments, isolated proteins or biologically active portions thereof lack contaminating proteins from the same animal from which the GFRα-X protein is derived. Typically, such proteins are produced by recombinant expression of, for example, a human GFRα-X protein in a non-human cell.

Preferably, an isolated GFRα-X protein or a portion thereof of the invention can bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell. In preferred embodiments, the protein or portion thereof comprises an amino acid sequence which is sufficiently homologous to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof maintains the ability to bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell. The portion of the protein is preferably a biologically active portion as described herein. In still another preferred embodiment, the GFRα-X protein has an amino acid sequence which is encoded by a nucleotide sequence that is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence of the DNA SEQ ID NO:1. The preferred GFRα-X proteins of the present invention also preferably possess at least one of the GFRα-X activities described herein.

In other embodiments, the GFRα-X protein is substantially homologous to the amino acid sequence of SEQ ID NO:2 and retains the functional activity of the protein of SEQ ID NO:2 yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail in subsection I above. Accordingly, in another embodiment, the GFRα-X protein is a protein which comprises an amino acid sequence which is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the entire amino acid sequence of SEQ ID NO:2 and which has at least one of the GFRα-X activities described herein. In other embodiment, the invention pertains to a protein which is substantially homologous to the entire amino acid sequence of SEQ ID NO:2.

Biologically active portions of the GFRα-X protein include peptides comprising amino acid sequences derived from the amino acid sequence of the GFRα-X protein, e.g., the amino acid sequence shown in SEQ ID NO:2 or the amino acid sequence of a protein homologous to the GFRα-X protein, which include less amino acids than the GFRα-X protein or the full length protein which is homologous to the GFRα-X protein, and exhibit at least one activity of the GFRα-X protein. Typically, biologically active portions (peptides, e.g., peptides which are, for example, 5, 10, 15, 20, 30, 35, 36, 37, 38, 39, 40, 50, 100 or more amino acids in length) comprise a domain or motif, e.g., an N-terminal hydrophobic domain, with at least one activity of the GFRα-X protein. Preferably, the domain is an N-terminal hydrophobic domain derived from a human and is at least about 55-60%, preferably at least about 65-70%, even more preferably at least about 75-80%, and most preferably at least about 85-90% or more homologous to SEQ ID NO:2.

GFRα-X proteins are preferably produced by recombinant DNA techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector (as described above), the expression vector is introduced into a host cell (as described above) and the GFRα-X protein is expressed in the host cell. The GFRα-X protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a GFR α-X protein, polypeptide, or peptide can be synthesized chemically using standard peptide synthesis techniques. Moreover, native GFRα-X protein can be isolated from cells (e.g., neural cells), for example using an anti-GFRα-X antibody (described further below).

The invention also provides GFRα-X chimeric or fusion proteins. As used herein, a GFRα-X "chimeric protein" or "fusion protein" comprises a GFRα-X polypeptide operatively linked to a non-GFRα-X polypeptide. An "GFRα-X polypeptide" refers to a polypeptide having an amino acid sequence corresponding to GFRα-X, or a fragment thereof, whereas a "non-GFRα-X polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not

substantially homologous to the GFRα-X protein, e.g., a protein which is different from the GFRα-X protein and which is derived from the same or a different organism. Within the fusion protein, the term "operatively linked" is intended to indicate that the GFRα-X polypeptide and the non-GFRα-X polypeptide are fused in-frame to each other. The non-GFRα-X polypeptide can be fused to the N-terminus or C-terminus of the GFRα-X polypeptide. For example, in one embodiment the fusion protein is a GST-GFRα-X fusion protein in which the GFRα-X sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant GFRα-X.

Preferably, a GFRα-X chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). A GFRα-X-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the GFRα-X protein.

The present invention also pertains to homologues of the GFRα-X proteins which function as either a GFRα-X agonist (mimetic) or a GFRα-X antagonist. In a preferred embodiment, the GFRα-X agonists and antagonists stimulate or inhibit, respectively, a subset of the biological activities of the naturally occurring form of the GFRα-X protein. Thus, specific biological effects can be elicited by treatment with a

homologue of limited function. In one embodiment, treatment of a subject with a homologue having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the GFRα-X protein.

Homologues of the GFRα-X protein can be generated by mutagenesis, e.g., discrete point mutation or truncation of the GFRα-X protein. As used herein, the term "homologue" refers to a variant form of the GFRα-X protein which acts as an agonist or antagonist of the activity of the GFRα-X protein. An agonist of the GFRα-X protein can retain substantially the same, or a subset, of the biological activities of the GFRα-X protein. An antagonist of the GFRα-X protein can inhibit one or more of the activities of the naturally occurring form of the GFRα-X protein, by, for example, competitively binding to a downstream or upstream member of the GFRα-X cascade which includes the GFRα-X protein. Thus, the mammalian GFRα-X protein and homologues thereof of the present invention can be either positive or negative regulators of neurotrophic factor responses in cells responsive to a neurotrophic factor.

In an alternative embodiment, homologues of the GFRα-X protein can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants, of the GFRα-X protein for GFRα-X protein agonist or antagonist activity. In one embodiment, a variegated library of GFRα-X variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of GFRα-X variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential GFRα-X sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of GFRα-X sequences therein. There are a variety of methods which can be used to produce libraries of potential GFRα-X homologues from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one

mixture, of all of the sequences encoding the desired set of potential GFRα-X sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of the GFRα-X protein coding can be used to generate a variegated population of GFRα-X fragments for screening and subsequent selection of homologues of a GFRα-X protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a GFRα-X coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the GFRα-X protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of GFR α-X homologues. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recrusive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify GFRα-X homologues (Arkin and Yourvan (1992) *PNAS 89*:7811-7815; Delgrave et al. (1993) *Protein Engineering* 6(3):327-331).

In one embodiment, cell based assays can be exploited to analyze a variegated GFRα-X library. For example, a library of expression vectors can be transfected into a cell line ordinarily responsive to a particular neurotrophic factor. The transfected cells are then contacted with the neurotrophic factor and the effect of the GFRα-X mutant on signaling by the neurotrophic factor can be detected, e.g., by measuring ^{3}[H]thymidine incorporation. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of neurotrophic factor induction, and the individual clones further characterized.

An isolated GFRα-X protein, or a portion or fragment thereof (particularly fragments comprising residues displaying high antigenicity scores, Fig. 3), can be used as an immunogen to generate antibodies that bind GFRα-X using standard techniques for polyclonal and monoclonal antibody preparation. The GFRα-X protein of SEQ ID NO:2 can be used or, alternatively, the invention provides antigenic peptide fragments of GFRα-X for use as immunogens. The antigenic peptide of GFRα-X comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of GFRα-X such that an antibody raised against the peptide forms a specific immune complex with GFRα-X. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of GFRα-X that are located on the surface of the protein, e.g., hydrophilic regions.

A GFRα-X immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed GFRα-X protein or a chemically synthesized GFRα-X peptide. Prefered fragments of GFRα-X for use as an immunogen are fragments comprising high antigenicity scores shown in Figue 2 and conserved regions of high homology shown in Figure 3.. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable

subject with an immunogenic GFRα-X preparation induces a polyclonal anti-GFRα-X antibody response.

Accordingly, another aspect of the invention pertains to anti-GFRα-X antibodies. Preferably the antibodies of the present invention will bind GFRα-X but will not bind GFRα-1, GFRα-2, or GFRα-3. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as GFRα-X. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind GFRα-X. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of GFRα-X. A monoclonal antibody composition thus typically displays a single binding affinity for a particular GFRα-X protein with which it immunoreacts.

Polyclonal anti-GFRα-X antibodies can be prepared as described above by immunizing a suitable subject with a GFRα-X immunogen. The anti-GFRα-X antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized GFRα-X. If desired, the antibody molecules directed against GFRα-X can be isolated from the mammal (e.g., from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-GFRα-X antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown et al. (1981) *J. Immunol.* 127:539-46; Brown et al. (1980) *J. Biol. Chem* .255:4980-83; Yeh et al. (1976) *PNAS* 76:2927-31; and Yeh et al. (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72),

the EBV-hybridoma technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter et al. (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a GFRα-X immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds GFRα-X.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-GFRα-X monoclonal antibody (see, e.g., G. Galfre et al. (1977) *Nature* 266:55052; Gefter et al. *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody

of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind GFRα-X, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-GFRα-X antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with GFRα-X to thereby isolate immunoglobulin library members that bind GFRα-X. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. PCT International Publication No. WO 92/18619; Dower et al. PCT International Publication No. WO 91/17271; Winter et al. PCT International Publication WO 92/20791; Markland et al. PCT International Publication No. WO 92/15679; Breitling et al. PCT International Publication WO 93/01288; McCafferty et al. PCT International Publication No. WO 92/01047; Garrard et al. PCT International Publication No. WO 92/09690; Ladner et al. PCT International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J. Mol. Biol.* 226:889-896; Clarkson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas et al. (1991) *PNAS* 88:7978-7982; and McCafferty et al. *Nature* (1990) 348:552-554.

Additionally, recombinant anti-GFRα-X antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson et al. International Application No. PCT/US86/02269; Akira, et al. European

Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al. European Patent Application 173,494; Neuberger et al. PCT International Publication No. WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al. European Patent Application 125,023; Better et al. (1988) *Science* 240:1041-1043; Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *PNAS* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi et al. (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060.

An anti-GFRα-X antibody (e.g., monoclonal antibody) can be used to isolate GFRα-X by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-GFRα-X antibody can facilitate the purification of natural GFRα-X from cells and of recombinantly produced GFRα-X expressed in host cells. Moreover, an anti-GFRα-X antibody can be used to detect GFRα-X protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the GFRα-X protein. Anti-GFRα-X antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent

materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

IV. Pharmaceutical Compositions

The *GFRα-X* nucleic acid molecules, GFRα-X proteins, fragments thereof, GFR α-X modulators, and anti-GFRα-X antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. Such compositions typically comprise the nucleic acid molecule, protein, modulator, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a GFRα-X protein, fragment, or anti-GFRα-X antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

V. Uses and Methods of the Invention

The nucleic acid molecules, proteins, protein homologues, modulators, and antibodies described herein can be used in one or more of the following methods: a) drug screening assays; b) diagnostic assays; c) methods of treatment; d) pharmacogenomics; and e) monitoring of effects during clinical trials. A GFRα-X protein of the invention has one or more of the activities described herein and can thus be used to, for example, bind a neurotrophic factor and modulate response in a neurotrophic factor responsive cell. The isolated nucleic acid molecules of the invention can be used to express GFRα-X protein (e.g., via a recombinant expression vector in a host cell in gene therapy applications), to detect GFRα-X encoding mRNA (e.g., in a biological sample) or a genetic mutation in a *GFRα-X* gene, and to modulate GFRα-X activity, as described further below. In addition, the GFRα-X proteins can be used to screen drugs or compounds which modulate GFRα-X protein activity as well as to treat disorders characterized by insufficient production of GFRα-X protein or production of GFRα-X protein forms which have decreased activity compared to wild type GFRα-X. Moreover, the anti-GFRα-X antibodies of the invention can be used to detect and isolate GFRα-X protein and modulate GFRα-X protein activity.

a. Drug Screening Assays

The invention provides methods for identifying compounds or agents that can be used to treat disorders characterized by (or associated with) aberrant or abnormal *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. These methods are also referred to herein as drug screening assays and typically include the step of screening a candidate/test compound or agent for the ability to interact with (e.g., bind to) a GFRα-X protein, to modulate the interaction of a GFRα-X protein and a target molecule, and/or to modulate *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. Candidate/test compounds or agents which have one or more of these abilities can be used as drugs to treat disorders characterized by aberrant or abnormal *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. Candidate/test compounds include, for

example, 1) peptides such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, e.g., Lam, K.S. et al. (1991) *Nature* 354:82-84; Houghten, R. et al. (1991) *Nature* 354:84-86) and combinatorial chemistry-derived molecular libraries made of D- and/or L- configuration amino acids; 2) phosphopeptides (e.g., members of random and partially degenerate, directed phosphopeptide libraries, see, e.g., Songyang, Z. et al. (1993) *Cell* 72:767-778); 3) antibodies (e.g., polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, $F(ab')_2$, Fab expression library fragments, and epitope-binding fragments of antibodies); and 4) small organic and inorganic molecules (e.g., molecules obtained from combinatorial and natural product libraries).

In one embodiment, the invention provides assays for screening candidate/test compounds which interact with (e.g., bind to) GFRα-X protein. Typically, the assays are cell-based assays which include the steps of combining a GFRα-X protein, a biologically active portion thereof, or a cell expressing GFRα-X protein or fragment thereof, and a candidate/test compound, e.g., under conditions which allow for interaction of (e.g., binding of) the candidate/test compound to the GFRα-X protein or portion thereof to form a complex, and detecting the formation of a complex, in which the ability of the candidate compound to interact with (e.g., bind to) the GFRα-X protein or portion thereof is indicated by the presence of the candidate compound in the complex. Formation of complexes between the GFRα-X protein and the candidate compound can be quantitated, for example, using standard immunoassays.

In another embodiment, the invention provides screening assays to identify candidate/test compounds which modulate (e.g., stimulate or inhibit) the interaction (and most likely GFRα-X activity as well) between a GFRα-X protein and a molecule (target molecule) with which the GFRα-X protein normally interacts. Examples of such target molecules includes proteins in the same signaling path as the GFRα-X protein, e.g., proteins which may function upstream (including both stimulators and inhibitors of activity) e.g., GDNF and/or NTN or downstream of the GFRα-X protein in the neurotrophic factor signaling pathway, e.g., the tyrosine kinase RET receptor. Typically, the assays are cell-based assays which include the steps of combining a GFR

α-X protein, a biologically active portion thereof, or a cell expressing GFRα-X protein or fragment thereof, a GFRα-X target molecule (e.g., a GFRα-X ligand) and a candidate/test compound, e.g., under conditions wherein but for the presence of the candidate compound e.g., GDNF or NTN, the GFRα-X protein or biologically active portion thereof interacts with (e.g., binds to) the target molecule, and detecting the formation of a complex which includes the GFRα-X protein and the target molecule or detecting the interaction/reaction of the GFRα-X protein and the target molecule. Detection of complex formation can include direct quantitation of the complex by, for example, measuring inductive effects of the GFRα-X protein. A statistically significant change, such as a decrease, in the interaction of the GFRα-X and target molecule (e.g., in the formation of a complex between the GFRα-X and the target molecule) in the presence of a candidate compound (relative to what is detected in the absence of the candidate compound) is indicative of a modulation (e.g., stimulation or inhibition) of the interaction between the GFRα-X protein and the target molecule. Modulation of the formation of complexes between the GFRα-X protein and the target molecule can be quantitated using, for example, an immunoassay.

To perform the above drug screening assays, it may be desirable to immobilize either GFRα-X or its target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Interaction (e.g., binding of) of GFRα-X to a target molecule, in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/ GFRα-X fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the cell lysates (e.g. ^{35}S-labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads are washed to remove any unbound label, and the matrix

immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of GFRα-X-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices can also be used in the drug screening assays of the invention. For example, either GFRα-X or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated GFRα-X molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with GFRα-X but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and GFRα-X trapped in the wells by antibody conjugation. As described above, preparations of a GFRα-X-binding protein and a candidate compound are incubated in the GFRα-X-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the GFRα-X target molecule, or which are reactive with GFRα-X protein and compete with the target molecule; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target molecule.

In yet another embodiment, the invention provides a method for identifying a compound (e.g., a screening assay) capable of use in the treatment of a disorder characterized by (or associated with) aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity. This method typically includes the step of assaying the ability of the compound or agent to modulate the expression of the *GFRα-X* nucleic acid or the activity of the GFRα-X protein thereby identifying a compound for treating a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity. Disorders characterized by aberrant or abnormal

GFRα-X nucleic acid expression or GFRα-X protein activity are described herein. Methods for assaying the ability of the compound or agent to modulate the expression of the *GFRα-X* nucleic acid or activity of the GFRα-X protein are typically cell-based assays. For example, cells which are sensitive to ligands, e.g., GDNF, which transduce signals via a pathway involving GFRα-X can be induced to overexpress a GFRα-X protein in the presence and absence of a candidate compound. Candidate compounds which produce a statistically significant change in GFRα-X-dependent responses (either stimulation or inhibition) can be identified. In one embodiment, expression of the *GFR α-X* nucleic acid or activity of a GFRα-X protein is modulated in cells and the effects of candidate compounds on the readout of interest (such as rate of cell proliferation or differentiation) are measured. For example, the expression of genes which are up- or down-regulated in response to a GFRα-X-dependent signal cascade can be assayed. In preferred embodiments, the regulatory regions of such genes, e.g., the 5' flanking promoter and enhancer regions, are operably linked to a detectable marker (such as luciferase) which encodes a gene product that can be readily detected. Phosphorylation of GFRα-X or GFRα-X target molecules can also be measured, for example, by immunoblotting.

Alternatively, modulators of GFRα-X expression (e.g., compounds which can be used to treat a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity) can be identified in a method wherein a cell is contacted with a candidate compound and the expression of GFRα-X encoding mRNA or protein in the cell is determined. The level of expression of GFRα-X encoding mRNA or protein in the presence of the candidate compound is compared to the level of expression of GFRα-X encoding mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of *GFRα-X* nucleic acid expression based on this comparison and be used to treat a disorder characterized by aberrant *GFRα-X* nucleic acid expression. For example, when expression of GFRα-X encoding mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate

compound is identified as a stimulator of GFRα-X encoding mRNA or protein expression. Alternatively, when expression of GFRα-X encoding mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of GFRα-X encoding mRNA or protein expression. The level of GFRα-X encoding mRNA or protein expression in the cells can be determined by methods described herein for detecting GFRα-X encoding mRNA or protein.

In yet another aspect of the invention, the GFRα-X proteins, particularly fragments of GFRα-X, can be used as "bait proteins" in a two-hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J. Biol. Chem.* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; Iwabuchi et al. (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with GFRα-X ("GFRα-X-binding proteins" or "GFR α-X-bp") and modulate GFRα-X protein activity. Such GFRα-X-binding proteins are also likely to be involved in the propagation of signals by the GFRα-X proteins as, for example, upstream or downstream elements of the GFRα-X pathway.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for GFRα-X is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming a GFRα-X-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcription regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with GFRα-X.

Modulators of GFRα-X protein activity and/or *GFRα-X* nucleic acid expression identified according to these drug screening assays can be to treat, for example, neurological diseases or disorders described herein. These methods of treatment include the steps of administering the modulators of GFRα-X protein activity and/or nucleic acid expression, e.g., in a pharmaceutical composition as described in subsection IV above, to a subject in need of such treatment, e.g., a subject with a neurological disease.

b. Diagnostic Assays

The invention further provides a method for detecting the presence of GFRα-X in a biological sample. The method involves contacting the biological sample with a compound or an agent capable of detecting GFRα-X protein or mRNA such that the presence of GFRα-X is detected in the biological sample. A preferred agent for detecting GFRα-X encoding mRNA is a labeled or labelable nucleic acid probe capable of hybridizing to GFRα-X encoding mRNA. The nucleic acid probe can be, for example, the *GFRα-X* cDNA of SEQ ID NO:1, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to GFRα-X encoding mRNA. A preferred agent for detecting GFRα-X protein is a labeled or labelable antibody capable of binding to GFRα-X protein. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) can be used. The term "labeled or labelable", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect GFRα-X encoding mRNA or protein in a

biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of GFRα-X encoding mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of GFRα-X protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. Alternatively, GFRα-X protein can be detected *in vivo* in a subject by introducing into the subject a labeled anti-GFRα-X antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques. In one preferred embodiment of the detection method, the biological sample is a neural cell sample. The neural cell sample can comprise neural tissue or a suspension of neural cells. A tissue section, for example, a freeze-dried or fresh frozen section of neural tissue removed from a patient, can be used as the neural cell sample. Alternatively, the biological sample can comprise a biological fluid (e.g., cerebrospinal fluid) obtained from a subject having a neurological disorder. In another preferred embodiment of the detection method, the biological sample is a neural cell sample (e.g., a sample which includes motorneuron cells). The invention also encompasses kits for detecting the presence of GFRα-X in a biological sample. For example, the kit can comprise a labeled or labelable compound or agent capable of detecting GFRα-X protein or mRNA in a biological sample; means for determining the amount of GFRα-X in the sample; and means for comparing the amount of GFRα-X in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect GFRα-X encoding mRNA or protein.

The methods of the invention can also be used to detect genetic mutations in a *GFRα-X* gene, or the allelic form of *GFRα-X* found in a subject, thereby determining if a subject with the mutated gene is at risk for a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity as defined herein. In preferred embodiments, the methods include detecting, in a sample from the subject, the presence or absence of a genetic mutation characterized by at least one of an alteration affecting the integrity of a gene encoding a GFRα-X protein, or the misexpression of the *GFRα-X* gene. For example, such genetic mutations can be

detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a *GFRα-X* gene; 2) an addition of one or more nucleotides to a *GFRα-X* gene; 3) a substitution of one or more nucleotides of a *GFRα-X* gene, 4) a chromosomal rearrangement of a *GFRα-X* gene; 5) an alteration in the level of a messenger RNA transcript of a *GFRα-X* gene, 6) aberrant modification of a *GFRα-X* gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a *GFRα-X* gene, 8) a non-wild type level of a GFRα-X-protein, 9) allelic loss of a *GFRα-X* gene, and 10) inappropriate post-translational modification of a GFRα-X-protein. As described herein, there are a large number of assay techniques known in the art which can be used for detecting mutations in a *GFRα-X* gene.

In certain embodiments, detection of the mutation involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) *Science* 241:1077-1080; and Nakazawa et al. (1994) *PNAS* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the GFRα-X-gene (see Abravaya et al. (1995) *Nucleic Acids Res* .23:675-682). This method can include the steps of collecting a sample from a patient, isolating nucleic acid (e.g., genomic, mRNA or both) from the sample, contacting the nucleic acid with one or more primers which specifically hybridize to a *GFRα-X* gene under conditions such that hybridization and amplification of the *GFRα-X*-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In an alternative embodiment, mutations in a *GFRα-X* gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence

specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the *GFRα-X* gene and detect mutations by comparing the sequence of the sample *GFRα-X* with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxim and Gilbert ((1977) *PNAS* 74:560) or Sanger ((1977) *PNAS* 74:5463). A variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO 94/16101; Cohen et al. (1996) *Adv. Chromatogr.* 36:127-162; and Griffin et al. (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the *GFRα-X* gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al. (1985) *Science* 230:1242); Cotton et al. (1988) *PNAS* 85:4397; Saleeba et al. (1992) *Meth. Enzymol.* 217:286-295), electrophoretic mobility of mutant and wild type nucleic acid is compared (Orita et al. (1989) *PNAS* 86:2766; Cotton (1993) *Mutat Res* 285:125-144; and Hayashi (1992) *Genet Anal Tech Appl* 9:73-79), and movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (Myers et al (1985) *Nature* 313:495). Examples of other techniques for detecting point mutations include, selective oligonucleotide hybridization, selective amplification, and selective primer extension.

c. Methods of Treatment

Another aspect of the invention pertains to methods for treating a subject, e.g., a human, having a disease or disorder characterized by (or associated with) aberrant or abnormal *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. These methods include the step of administering a GFRα-X modulator to the subject such that

treatment occurs. The language "aberrant or abnormal *GFRα-X* expression" refers to expression of a non-wild-type GFRα-X protein or a non-wild-type level of expression of a GFRα-X protein. Aberrant or abnormal GFRα-X activity refers to a non-wild-type GFRα-X activity or a non-wild-type level of GFRα-X activity. As the GFRα-X protein is involved in a pathway involving neurological and developmental functions, aberrant or abnormal GFRα-X protein activity or nucleic acid expression interferes with normal neurological functions and/or developmental functions. Non-limiting examples of neurological disorders or diseases characterized by or associated with abnormal or aberrant GFRα-X protein activity or nucleic acid expression in neural cells include sensory, disorders, e.g., Dejerine-Roussy Syndrome, motor disorders, e.g., Parkinson's disease, ALS, and cognitive disorders, e.g., Alzheimer's disease. Examples of disorders or diseases characterized by or associated with abnormal or aberrant GFRα-X protein activity or nucleic acid expression in cells associated with developmental function include disorders of the enteric nervous system, e.g., Hirschsprung's disease and eating disorders.

The terms "treating" or "treatment", as used herein, refer to reduction or alleviation of at least one adverse effect or symptom of a disorder or disease, e.g., a disorder or disease characterized by or associated with abnormal or aberrant GFRα-X protein activity or *GFRα-X* nucleic acid expression.

As used herein, a GFRα-X modulator is a molecule which can modulate *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. For example, a GFRα-X modulator can modulate, e.g., upregulate (activate) or downregulate (suppress), *GFRα-X* nucleic acid expression. In another example, a GFRα-X modulator can modulate (e.g., stimulate or inhibit) GFRα-X protein activity. If it is desirable to treat a disorder or disease characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFRα-X* nucleic acid expression and/or GFRα-X protein activity by inhibiting *GFRα-X* nucleic acid expression, a GFRα-X modulator can be an antisense molecule, e.g., a ribozyme, as described herein. Examples of antisense molecules which can be used to inhibit *GFRα-X* nucleic acid expression include antisense molecules which are

complementary to a portion of the 5' untranslated region of the GFRα-X encoding sequence which also includes the start codon and antisense molecules which are complementary to a portion of the 3' untranslated region.

A GFRα-X modulator which inhibits *GFRα-X* nucleic acid expression can also be a small molecule or other drug, e.g., a small molecule or drug identified using the screening assays described herein, which inhibits *GFRα-X* nucleic acid expression. If it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFRα-X* nucleic acid expression and/or GFRα-X protein activity by stimulating *GFRα-X* nucleic acid expression, a GFRα-X modulator can be, for example, a nucleic acid molecule encoding GFRα-X (e.g., a nucleic acid molecule comprising a nucleotide sequence homologous to the nucleotide sequence of SEQ ID NO:1) or a small molecule or other drug, e.g., a small molecule (peptide) or drug identified using the screening assays described herein, which stimulates *GFRα-X* nucleic acid expression.

Alternatively, if it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFRα-X* nucleic acid expression and/or GFRα-X protein activity by inhibiting GFRα-X protein activity, a GFRα-X modulator can be an anti-GFRα-X antibody or a small molecule or other drug, e.g., a small molecule or drug identified using the screening assays described herein, which inhibits GFRα-X protein activity. If it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFRα-X* nucleic acid expression and/or GFRα-X protein activity by stimulating GFRα-X protein activity, a GFRα-X modulator can be an active GFRα-X protein or portion thereof (e.g., a GFRα-X protein or portion thereof having an amino acid sequence which is homologous to the amino acid sequence of SEQ ID NO:2 or a portion thereof) or a small molecule or other drug, e.g., a small molecule or drug identified using the screening assays described herein, which stimulates GFRα-X protein activity.

In addition, a subject having a neurological disorder can be treated according to the present invention by administering to the subject a GFRα-X protein, preferably a portion thereof, or a nucleic acid encoding a GFRα-X protein or portion thereof such that treatment occurs. Similarly, a subject having a developmental disorder can be treated according to the present invention by administering to the subject a GFRα-X protein or portion thereof or a nucleic acid encoding a GFRα-X protein or portion thereof such that treatment occurs.

Other aspects of the invention pertain to methods for modulating a cell associated activity. These methods include contacting the cell with an agent (or a composition which includes an effective amount of an agent) which modulates GFRα-X protein activity or *GFRα-X* nucleic acid expression such that a cell associated activity is altered relative to a cell associated activity of the cell in the absence of the agent. As used herein, "a cell associated activity" refers to a normal or abnormal activity or function of a cell. Examples of cell associated activities include proliferation, migration, differentiation, production or secretion of molecules, such as proteins, and cell survival. In a preferred embodiment, the cell is neural cell of the CNS, e.g., motorneuron of the spinal cord. The term "altered" as used herein refers to a change, e.g., an increase or decrease, of a cell associated activity. In one embodiment, the agent stimulates GFRα-X protein activity or *GFRα-X* nucleic acid expression. Examples of such stimulatory agents include an active GFRα-X protein, a nucleic acid molecule encoding GFRα-X that has been introduced into the cell, and a modulatory agent which stimulates GFR-Xα protein activity or *GFRα-X* nucleic acid expression and which is identified using the drug screening assays described herein. In another embodiment, the agent inhibits GFR α-X protein activity or *GFRα-X* nucleic acid expression. Examples of such inhibitory agents include an antisense *GFRα-X* nucleic acid molecule, an anti-GFR-Xα antibody, and a modulatory agent which inhibits GFRα-X protein activity or *GFRα-X* nucleic acid expression and which is identified using the drug screening assays described herein. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject). In a preferred embodiment, the modulatory methods are performed *in vivo*, i.e., the cell is

present within a subject, e.g., a mammal, e.g., a human, and the subject has a disorder or disease characterized by or associated with abnormal or aberrant GFRα-X protein activity or *GFRα-X* nucleic acid expression.

A nucleic acid molecule, a protein, a GFRα-X modulator, a compound etc. used in the methods of treatment can be incorporated into an appropriate pharmaceutical composition described herein and administered to the subject through a route which allows the molecule, protein, modulator, or compound etc. to perform its intended function. Examples of routes of administration are also described herein under subsection IV.

d. Pharmacogenomics

Test/candidate compounds, or modulators which have a stimulatory or inhibitory effect on GFRα-X activity (e.g., *GFRα-X* gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (e.g., neural disorders, e.g., central and peripheral nervous system disorders) associated with aberrant GFRα-X activity. In conjunction with such treatment, the pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permit the selection of effective compounds (e.g., drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly, the activity of GFRα-X polypeptide, expression of *GFRα-X* nucleic acid, or mutation content of *GFRα-X* genes in an individual can be determined to thereby select appropriate compound(s) for therapeutic or prophylactic treatment of the individual.

Pharmacogenomics deal with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Eichelbaum, M. (1996) *Clin. Exp. Pharmacol. Physiol.* 23(10-11):983-985 and Linder, M.W. (1997) *Clin. Chem.* 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus, the activity of GFRα-X polypeptide, expression of *GFRα-X* nucleic acid, or mutation content of *GFRα-X* genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of a subject. In addition, pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of a subject's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with an GFRα-X modulator, such as a modulator identified by one of the exemplary screening assays described herein.

e. Monitoring of Effects During Clinical Trials

Monitoring the influence of compounds (e.g., drugs) on the expression or activity of GFRα-X (e.g., the ability to modulate the effects of neurotrophic factors on neurotrophic factor responsive cells) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay, as described herein, to increase *GFRα-X* gene expression, polypeptide levels, or up-regulate GFRα-X activity, can be monitored in clinical trials of subjects exhibiting decreased *GFRα-X* gene expression, polypeptide levels, or down-regulated GFRα-X activity. Alternatively, the effectiveness of an agent, determined by a screening assay, to decrease *GFRα-X* gene expression, polypeptide levels, or down-regulate GFRα-X activity, can be monitored in clinical trials of subjects exhibiting increased *GFRα-X* gene expression, polypeptide levels, or up-regulated GFRα-X activity. In such clinical trials, the expression or activity of GFRα-X and, preferably, other genes which have been implicated in, for example, a neural disorder, e.g., a central nervous system disorder, can be used as a "read out" or markers of the neurotrophic factor responsiveness of a particular cell.

For example, and not by way of limitation, genes, including GFRα-X, which are modulated in cells by treatment with a compound (e.g., drug or small molecule) which modulates GFRα-X activity (e.g., identified in a screening assay as described herein) can be identified. Thus, to study the effect of compounds on neural disorders, e.g.,

central and peripheral nervous system disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of GFRα-X and other genes implicated in the disorder. The levels of gene expression (i.e., a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of polypeptide produced, by one of the methods described herein, or by measuring the levels of activity of GFRα-X or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the compound. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the compound.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with a compound (e.g., an agonist, antagonist, peptidomimetic, polypeptide, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the compound; (ii) detecting the level of expression of an GFRα-X polypeptide, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the GFRα-X polypeptide, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the GFRα-X polypeptide, mRNA, or genomic DNA in the pre-administration sample with the GFRα-X polypeptide, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the compound to the subject accordingly. For example, increased administration of the compound may be desirable to increase the expression or activity of GFRα-X to higher levels than detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of GFRα-X to lower levels than detected, i.e., to decrease the effectiveness of the compound.

VI. Uses f Partial GFRα-X Sequences

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (a) map their respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (b) identify an individual from a minute biological sample (tissue typing); and (c) aid in forensic identification of a biological sample. These applications are described in the subsections below.

a. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. This process is called chromosome mapping. Accordingly, portions or fragments of the GFRα-X sequence, described herein, can be used to map the location of the *GFRα-X* gene, respectively, on a chromosome. The mapping of the *GFRα-X* sequence to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, the *GFRα-X* gene can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the GFRα-X sequences. Computer analysis of the

GFRα-X sequence can be used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the GFRα-X sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (e.g., human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains

the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio P. et al. (1983) *Science* 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the GFRα-X sequence to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a GFRα-X sequence to its chromosome include *in situ* hybridization (described in Fan, Y. et al. (1990) *PNAS*, 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical like colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma et al., Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York 1988).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to

noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. et al. (1987) *Nature* 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the *GFRα-X* gene can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

b. Tissue Typing

The GFRα-X sequence of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the GFRα-X sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The GFRα-X sequence of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1 can comfortably provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If the predicted coding sequence, such as the one in SEQ ID NO:2 is used, a more appropriate number of primers for positive individual identification would be 500-2,000.

If a panel of reagents from GFRα-X sequence described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

c. Use of Partial GFRα-X Sequence in Forensic Biology

DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, e.g., PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (i.e., another DNA sequence that is unique to a particular individual). As discussed above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the GFRα-X sequence or portions thereof, e.g., fragments derived from the noncoding regions of SEQ ID NO:1 having a length of at least 20 bases, preferably at least 30 bases.

The GFRα-X sequences described herein can further be used to provide polynucleotide reagents, e.g., labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, e.g., brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such GFRα-X probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, e.g., GFRα-X primers or probes can be used to screen tissue culture for contamination (i.e., screen for the presence of a mixture of different types of cells in a culture).

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patent applications, patents, and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

EXAMPLE 1: IDENTIFICATION OF MOUSE *GFRα-X* CDNA

In this example, the GFRα-X mouse cDNA was identified in a positional cloning process in which the mouse mahogany locus was being sequenced.

EXAMPLE 2: IDENTIFICATION OF HUMAN *GFRα-X* cDNA

To obtain the human *GFRα-X* nucleic acid molecule, a cDNA library from a human brain cell library (available from Stratagene, LaJolla, CA, or Clontech, Palo Alto, CA) is screened under low stringency conditions (e.g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) using a probe comprising the nucleotide sequence of SEQ ID NO:1 or a portion thereof. Clones obtained from this screen are sequenced and compared to the mouse sequence shown in SEQ ID NO:1 to determine if they are the human GFRα-X molecule. If the clones are found to be partial clones, the cDNA library is rescreened with the partial human clone to obtain the full length human clone.

EXAMPLE 3:TISSUE EXPRESSION OF THE MOUSE GRFα-X GENE

Northern Analysis Using RNA from Human Tissue

Mouse multiple tissue northern blots (Stratagene, Palo Alto, CA) containing 2 μg of poly A+ RNA per lane were probed with probes based on SEQ ID No:1. The filters were prehybridized in 5 ml of Church buffer at 65°C for 1 hour, after which 100 ng of ^{32}P labeled probe was added. The probe was generated using the Stratagene Prime-It kit, Catalog Number 300392 (Clontech, Palo Alto, CA). Hybridization was allowed to

proceed at 65°C for approximately 20 hours. The filters were washed in 0.1% SDS, 0.2 X SSC solution at 65°C and then exposed to the phosphoimager for 4 hours. The rat tissues tested included: heart, brain, spleen, lung, liver, stomach, kidney, and testis.

There was strong hybridization to the brain RNA represented in this Northern blot indicating that the *GFRα-X* gene transcript is expressed in brain. In situ hybridization showed expression in the Lateral septal neurons, Septohypothalamic neurons, paraventricular thalamic neurons (anterior), superchiasmatic neurons, anterior cortical amygdaloid neurons, piriform cortex, paracentral thalamic neurons, lateral habenular neurons, paraventricular hypothalamic neurons (PVN), amygdaloid nucleus area, arcuate neurons, and ventromedial hypothalamic neurons (VMH).

EXAMPLE 4: EXPRESSION OF RECOMBINANT GFRα-X PROTEIN IN COS CELLS

To express the *GFRα-X* gene in COS cells, the pcDNA/Amp vector by Invitrogen Corporation (San Diego, CA) is used. This vector contains an SV40 origin of replication, an ampicillin resistance gene, an *E. coli* replication origin, a CMV promoter followed by a polylinker region, and an SV40 intron and polyadenylation site. A DNA fragment encoding the entire GFRα-X protein and a HA tag (Wilson et al. (1984) *Cell* 37:767) fused in-frame to its 3' end of the fragment is cloned into the polylinker region of the vector, thereby placing the expression of the recombinant protein under the control of the CMV promoter.

To construct the plasmid, the GFRα-X DNA sequence is amplified by PCR using two primers. The 5' primer contains the restriction site of interest followed by approximately twenty nucleotides of the GFRα-X coding sequence starting from the initiation codon; the 3' end sequence contains complementary sequences to the other restriction site of interest, a translation stop codon, the HA tag and the last 20 nucleotides of the GFRα-X coding sequence. The PCR amplified fragment and the pCDNA/Amp vector are digested with the appropriate restriction enzymes and the vector is dephosphorylated using the CIAP enzyme (New England Biolabs, Beverly, MA). Preferably the two restriction sites chosen are different so that the *GFRα-X* gene

is inserted in the correct orientation. The ligation mixture is transformed into *E. coli* cells (strains HB101, DH5a, SURE, available from Stratagene Cloning Systems, La Jolla, CA, can be used), the transformed culture is plated on ampicillin media plates, and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment.

COS cells are subsequently transfected with the GFRα-X-pcDNA/Amp plasmid DNA using the calcium phosphate or calcium chloride co-precipitation methods, DEAE-dextran-mediated transfection, lipofection, or electroporation. Other suitable methods for transfecting host cells can be found in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The expression of the GFRα-X protein is detected by radiolabelling (^{35}S-methionine or ^{35}S-cysteine available from NEN, Boston, MA, can be used) and immunoprecipitation (Harlow, E. and Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988) using an HA specific monoclonal antibody. Briefly, the cells are labeled for 8 hours with ^{35}S-methionine (or ^{35}S-cysteine). The culture media are then collected and the cells are lysed using detergents (RIPA buffer, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM Tris, pH 7.5). Both the cell lysate and the culture media are precipitated with an HA specific monoclonal antibody. Precipitated proteins are then analyzed by SDS-PAGE.

Alternatively, DNA containing the GFRα-X coding sequence is cloned directly into the polylinker of the pCDNA/Amp vector using the appropriate restriction sites. The resulting plasmid is transfected into COS cells in the manner described above, and the expression of the GFRα-X protein is detected by radiolabelling and immunoprecipitation using a GFRα-X specific monoclonal antibody

EXAMPLE 5: CHARACTERIZATION OF GFRα-X PROTEIN

In this example, the amino acid sequence of the GFRα-X protein was compared to amino acid sequences of known proteins and various motifs were identified.

The GFRα-X protein, the amino acid sequence of which is shown in Figure 1 (SEQ ID NO:2), is a novel protein which includes 340 amino acid residues. A comparison of GFRα-X with other members of the GFR family of proteins is provided in Figure 3.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. An isolated nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule which encodes a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a nucleic acid molecule which encodes a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a nucleic acid molecule which encodes a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:2 under stringent conditions.

2. The nucleic acid molecule of claim 1 further comprising vector nucleic acid sequences.

3. The nucleic acid molecule of claim 1 further comprising nucleic acid sequences encoding a heterologous protein.

4. A host cell which contains the nucleic acid molecule of claim 1.

5. The host cell of claim 4 which is a mammalian host cell.

6. A non-human mammalian host cell containing the nucleic acid molecule of claim 1.

7. The isolated nucleic acid molecule of claim 1, which is selected from the group consisting of the coding region of SEQ ID NO:1 and the extracellular domain encoded by SEQ ID NO:1.

8. An isolated protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions.

9. The protein of claim 8 further comprising heterologous amino acid sequences.

10. An antibody which selectively binds to a protein of claim 8.

11. A method for producing a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions;

the method comprising the step of culturing the host cell of claim 4 under conditions in which the nucleic acid molecule is expressed.

12. A method for detecting the presence of a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions;

in a sample, the method comprising the steps of:

i) contacting the sample with a compound which selectively binds to the protein; and

ii) determining whether the compound binds to the protein in the sample.

13. The method of claim 12, wherein the compound which binds to the protein is an antibody.

14. A kit comprising reagents used for the method of claim 12, wherein the reagents comprise a compound which selectively binds to a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a peptide comprising at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions.

15. A method for detecting the presence of a nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule which encodes a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a nucleic acid molecule which encodes a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a nucleic acid molecule which encodes a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions;

in a sample, the method comprising the steps of:

i) contacting the sample with a nucleic acid probe or primer which selectively hybridizes to the nucleic acid molecule; and

ii) determining whether the nucleic acid probe or primer binds to a nucleic acid molecule in the sample.

16. The method of claim 15, wherein the sample comprises mRNA molecules and is contacted with a nucleic acid probe.

17. A kit comprising reagents used for the method of claim 15, wherein the reagents comprise a compound which selectively hybridizes to a nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule which encodes a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a nucleic acid molecule which encodes a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a nucleic acid molecule which encodes a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions.

18. A method for identifying a compound which binds to a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions,

the method comprising the steps of:

i) contacting the protein, or a cell expressing the protein with a test compound; and

ii) determining whether the protein binds to the test compound.

19. The method of claim 18, wherein the binding of the test compound to the protein is detected by a method selected from the group consisting of:

a) detection of binding by direct detecting of test compound/protein binding;

b) detection of binding using a competition binding assay;

c) detection of binding using an assay for NT2LP activity.

20. A method for modulating the activity of a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2; and

b) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions,

the method comprising the steps of:

i) contacting a cell expressing the protein with a compound which binds to the protein in a sufficient concentration to modulate the activity of the protein.

TGTGGAATGTGGAGAACCAAGCACGGAGGACTGCAGCCTGCCCGCCCTTC

ACCAGGGTCTGCGAGCTTTACCGACGGGAATCGCTGCGTGGACGCGGCCG

AGGCGTGTACAGCAGACGAGCGGTGCCAGCAGCTGCGCTCTGAGTACGTG

GCACGATGCCTGGGCCGGGCAGCGCCCGGGGGCAGGCCGGGACCCGGGGG

CTGCGTGCGCTCCCGCTGCCGCCGACCCCTGCGCCGCTTCTTCGCGCGTG

GGCCTCCGGCGCTCACGCATGCGCTGCTCTTCTGCGGCTGCGAAGGCTCC

GCGTGCGCCGAGCGCCGGCGCCAGACTTTCGCGCCCGCCTGCGCGTTCTC

CGGCCCGGGGTTGGTGCCGCCCTCTTGCCTGGAGCCCCTGGAGCGCTGCG

AGCGCAGCCGCCTGTGCCGGCCCCGTCTCCTTGCCTTCCAGGCCTCATGC

GCTCCCGCGCCCGGCTCCCGCGACCGCTGCCCGGAGGAGGGGGGCCCGCG

TTGTCTGCGCGTCTACGCAGGCCTCATAGGCACCGTGGTCACCCCCAACT

ACCTGGACAACGTGAGCGCGCGCGTTGCGCCCTGGTGCGGCTGTGCGGCC

ANGTGGAAACCGGCGCGAAGAATGCGAAGCCTTCCGCAAGCTCTTTACAA

NGGAACCCCTGCTTGGGTGAGGGGGCCTGGAGGTCCCGGGGAACCACGGA

TGTCTGTGGCCCAATCCAAGCTGCCTGGCCCGTGGGTCTTATTTACGTCG

CATCATGTTTGGTGTGGGCGATGGACAGTGTGCACATGCCATGATGGTGC

CATACAAGCCTTTGACAGCTTGCAGCCATCAGTTCTGCAGGACCAGACTG

CTGGGTGCTGTTTCCCGCGGGCAAGGCACGAGTGGCCTGAGAAGAGCTGG

AGGCAGAAACAGTCCTTGTTTTGTCCTAACGCCCAAGGTGTCCTGGCTGT

ATGCACTCACTGCCCTGGCTCTCCAGGCCCTGCTCTGATTAGGAACATGA

ACCGTGGACGACACAGCTG

CGMWRTKHGGLQPARPSPGSASFTDGNRCVDAAEACTADERCQQLRSEYVARCLGRAAPG

GRPGPGGCVRSRCRRPLRRFFARGPPALTHALLFCGCEGSACAERRRQTFAPACAFSGPG

LVPPSCLEPLERCERSRLCRPRLLAFQASCAPAPGSRDRCPEEGGPRCLRVYAGLIGTVV

TPNYLDNVSARVAPWCGCAAXWKPARRMRSLPQALYXGTPAWVRGPGGPGEPRMSVAQSK

LPGPWVLFTSHHVWCGRWTVCTCHDGAIQAFDSLQPSVLQDQTAGCCFPRARHEWPEKSW

RQKQSLFCPNAQGVLAVCTHCPGSPGPALIRNMNRGRHSX

FIGURE 1

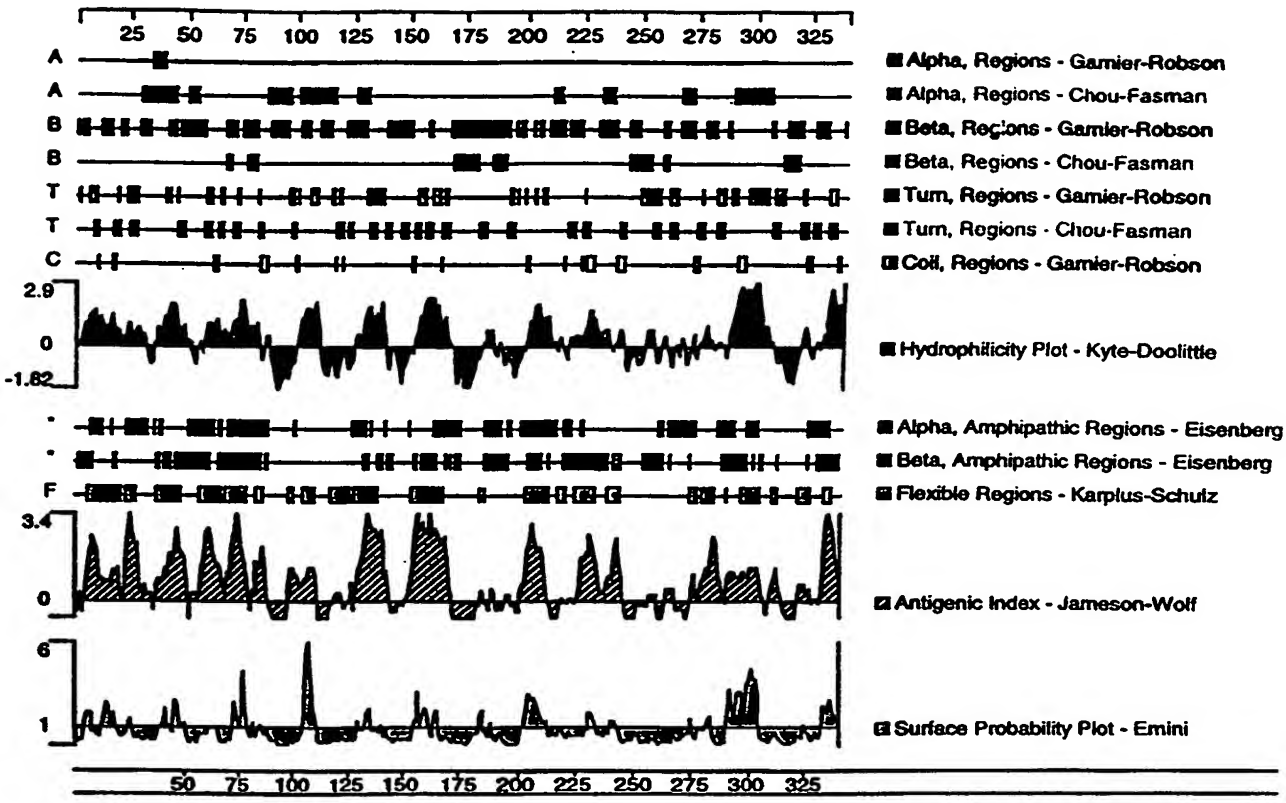

FIGURE 2

```
O08842NRTRmouse     ..........  .MILANAFCL FFFLDETLRS LASPSSPQGS ELHGWRPQVD
P97785GDNFRamouse   ..........  .MFLATLYFV LPLLDLLMSA EVS.....G. ...GDR..LD
AF045162chickGDNF4  ..........  .MRGILYFCT LILLEGMAEA VSS.......  ....SR...D
AF020305GDNFRa3mouse MGLSWSPRPP LLMILLLVLS LWLPLGAGNS LAT.......  ...ENRFVNS
gdnfrorfaa          ..........  ..........  ..........  ..........  ..........

O08842NRTRmouse     CVRANELCAA ESNCSSRYRT LRQCLAGRDR NTMLAN....  .KECQAALEV
P97785GDNFRamouse   CVKASDQCLK EQSCSTKYRT LRQCVAGKET NFSLTSGLEA KDECRSAMEA
AF045162chickGDNF4  CLQAGESCTN DPICSSKFRT LRQCIAGNGA NKLGPD...A KNQCRSTVTA
AF020305GDNFRa3mouse CTQARKKCEA NPACKAAYQH LGSCTSSLSR PLPLEES.AM SADCLEAAEQ
gdnfrorfaa          ..........  ..........  ..........  ..........  ..........

O08842NRTRmouse     LQESPLYDCR CKRGMKKELQ CLQIYWSIHL GLTEGEEFYE ASPYEPVTSR
P97785GDNFRamouse   LKQKSLYNCR CKRGMKKEKN CLRIYWSMYQ SLQ.GNDLLE DSPYEPVNSR
AF045162chickGDNF4  LLSSQLYGCK CKRGMKKEKH CLSVYWSIHH TLMEGMNVLE SSPYEPF.IR
AF020305GDNFRa3mouse LRNSSLIDCR CHRRMKHQAT CLDIYWTVHP ARSLGDYELD VSPYEDT.VT
gdnfrorfaa          ..........  ..........  CG.MWRTKHG GLQ.......  ..PARPS...

O08842NRTRmouse     LSDIFRLASI FSGTGADPVV SAKSNHCLDA AKACNLNDNC KKLRSSYISI
P97785GDNFRamouse   LSDIFRAVPF ISDVFQQVEH ISKGNNCLDA AKACNLDDTC KKYRSAYITP
AF045162chickGDNF4  GFDYVRLASI TAGSENE...  VTQVNRCLDA AKACNVDEMC QRLRTEYVSF
AF020305GDNFRa3mouse SKPWKMNLSK LNMLKPD...  ...SDLCLKF AMLCTLHDKC DRLRKAYGEA
gdnfrorfaa          .....PGSA SFTD......  ...GNRCVDA AEACTADERC QQLRSEYVAR

O08842NRTRmouse     CNREISP...  ..TERCNRRK CHKALRQFFD RVPSEYTYRM LFCSC..QDQ
P97785GDNFRamouse   CTTSMS....  ..NEVCNRRK CHKALRQFFD KVPAKHSYGM LFCSC..RDV
AF045162chickGDNF4  CIRRLAR...  ..ADTCNRSK CHKALRKFFD RVPPEYTHEL LFCPC..EDT
AF020305GDNFRa3mouse CS.........  ..GIRCQRHL CLAQLRSFFE KAAESHAQGL LLCPCPPEDA
gdnfrorfaa          CLGRAAPGGR PGPGGCVRSR CRRPLRRFFA RGPPALTHAL LFCGC..EGS

O08842NRTRmouse     ACAERRRQTI LPSCSYEDK.  .EKPNCLDLR SLCRTDHLCR SRLADFHANC
P97785GDNFRamouse   ACTERRRQTI VPVCSYEER.  .ERPNCLNLQ DSCKTNYICR SRLADFFTNC
AF045162chickGDNF4  ACAERRRQTI VPACSYESK.  .EKPNCLAPL DSCRENYVCR SRYAEFQFNC
AF020305GDNFRa3mouse GCGERRRNTI APSCALPS..  .VTPNCLDLR SPCRADPLCR SRLMDFQTHC
gdnfrorfaa          ACAERRRQTF APACAFSGPG LVPPSCLEPL ERCERSRLCR PRLLAFQASC

O08842NRTRmouse     RASYRTITSC PADNYQACLG SYAGMIGFDM TPNYVDSNPT GIVVSPWCNC
P97785GDNFRamouse   QPESRSVSNC LKENYADCLL AYSGLIGTVM TPNYIDS..S SLSVAPWCDC
AF045162chickGDNF4  QPSLQTASGC RRDSYAACLL AYTGIIGSPI TPNYIDN..S TSSIAPWCTC
AF020305GDNFRa3mouse HP.MDILGTC ATE.QSRCLR AYLGLIGTAM TPNFISK..V NTTVALSCTC
gdnfrorfaa          APAPGSRDRC PEEGGPRCLR VYAGLIGTVV TPNYLDN..V SARVAPWCGC

O08842NRTRmouse     RGSGNMEEEC EKFLKDFTEN PCLRNAIQAF GNGTDVNMSP KGP.....TF
P97785GDNFRamouse   SNSGNDLEDC LKFLNFFKDN TCLKNAIQAF GNGSDVTMWQ PAPPVQTTTA
AF045162chickGDNF4  NASGNRQEEC ESFLHLFTDN VCLQNAIQAF GNGTYLNAAT APS.....IS
AF020305GDNFRa3mouse RGSGNLQDEC EQLERSFSQN PCLVEAIAAK MRFHRQLFSQ DWA.....DS
gdnfrorfaa          AAXWKPARRM RSLPQALYXG TPAWVRGPGG PGEPRMSVAQ SKLPG..PWV

O08842NRTRmouse     SATQAPRVEK TPSLPDDLSD STSLGTSVIT TCTSIQEQ..  GLKANNSKEL
P97785GDNFRamouse   MTTTAFRIKN KPLGP..AGS ENEIPTHVLP PCANLQAQ..  KLKSNVSGST
AF045162chickGDNF4  PTTQMYKQER NANRAA.ATL SENIFEHLQP TKVAGEER..  LLRGSTRLSS
AF020305GDNFRa3mouse TFSVVQQQNS NPALR..LQP RLPILSFSIL PLILLQTL..  W.........
gdnfrorfaa          LFTSHHVWCG RWTVCTCHDG AIQAFDSLQP SVLQDQTAGC CFPRARHEWP
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FIGURE 3

```
O08842NRTRmouse              SMCFTELTTN ISPGSKKVIK LYSGSCRARL STALTALP.. .......LLM
P97785GDNFRamouse            HLCLSDNDYG KDGLAGASSH ITTKSMAAPP SCGLSSLPVM VFTALAALLS
AF045162chickGDNF4           ...ETSSPAA PCHQAASLLQ LWLPPTLAVL SHFMM..... ..........
AF020305GDNFRa3mouse         .......... .......... .......... .......... ..........
gdnfrorfaa                   EKSWRQKQSL PCPNAQGVLA VCTHCPGSPG PALIRNMN.. .......RGR

O08842NRTRmouse              VTLA...
P97785GDNFRamouse            VSLAETS
AF045162chickGDNF4           .......
AF020305GDNFRa3mouse         .......
gdnfrorfaa                   HSX....
```

FIGURE 3 (Cont'd)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MILLENNIUM PHARMACEUTICALS, INC.
(B) STREET: 640 MEMORIAL DRIVE
(C) CITY: CAMBRIDGE
(D) STATE: MASSACHUSETTS
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 02139
(G) TELEPHONE:
(H) TELEFAX:

(ii) TITLE OF INVENTION: GFRα-X, A NOVEL GLIAL-DERIVED NEUROTROPHIC FACTOR RECEPTOR AND USES THEREFOR

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LAHIVE & COCKFIELD, LLP
(B) STREET: 28 STATE STREET
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: US
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US99/
(B) FILING DATE: 25 MARCH 1999
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/080,070
(B) FILING DATE: 31 MARCH 1998

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MANDRAGOURAS, AMY E.
(B) REGISTRATION NUMBER: 36,207
(C) REFERENCE/DOCKET NUMBER: MNI-021PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617)227-7400
(B) TELEFAX: (617)742-4214

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1019 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1017

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TGT GGA ATG TGG AGA ACC AAG CAC GGA GGA CTG CAG CCT GCC CGC CCT      48
Cys Gly Met Trp Arg Thr Lys His Gly Gly Leu Gln Pro Ala Arg Pro
  1               5                  10                  15

TCA CCA GGG TCT GCG AGC TTT ACC GAC GGG AAT CGC TGC GTG GAC GCG      6
Ser Pro Gly Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala
            20                  25                  30

GCC GAG GCG TGT ACA GCA GAC GAG CGG TGC CAG CAG CTG CGC TCT GAG     144
Ala Glu Ala Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu
        35                  40                  45

TAC GTG GCA CGA TGC CTG GGC CGG GCA GCG CCC GGG GGC AGG CCG GGA     192
Tyr Val Ala Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly
    50                  55                  60

CCC GGG GGC TGC GTG CGC TCC CGC TGC CGC CGA CCC CTG CGC CGC TTC     240
Pro Gly Gly Cys Val Arg Ser Arg Cys Arg Arg Pro Leu Arg Arg Phe
65                  70                  75                  80

TTC GCG CGT GGG CCT CCG GCG CTC ACG CAT GCG CTG CTC TTC TGC GGC     288
Phe Ala Arg Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly
                85                  90                  95

TGC GAA GGC TCC GCG TGC GCC GAG CGC CGG CGC CAG ACT TTC GCG CCC     336
Cys Glu Gly Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro
            100                 105                 110

GCC TGC GCG TTC TCC GGC CCG GGG TTG GTG CCG CCC TCT TGC CTG GAG     384
Ala Cys Ala Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu
        115                 120                 125

CCC CTG GAG CGC TGC GAG CGC AGC CGC CTG TGC CGG CCC CGT CTC CTT     432
Pro Leu Glu Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu
    130                 135                 140
```

```
GCC TTC CAG GCC TCA TGC GCT CCC GCG CCC GGC TCC CGC GAC CGC TGC     480
Ala Phe Gln Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys
145                 150                 155                 160

CCG GAG GAG GGG GGC CCG CGT TGT CTG CGC GTC TAC GCA GGC CTC ATA     528
Pro Glu Glu Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile
                165                 170                 175

GGC ACC GTG GTC ACC CCC AAC TAC CTG GAC AAC GTG AGC GCG CGC GTT     576
Gly Thr Val Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val
            180                 185                 190

GCG CCC TGG TGC GGC TGT GCG GCC ANG TGG AAA CCG GCG CGA AGA ATG     624
Ala Pro Trp Cys Gly Cys Ala Ala Xaa Trp Lys Pro Ala Arg Arg Met
        195                 200                 205

CGA AGC CTT CCG CAA GCT CTT TAC AAN GGA ACC CCT GCT TGG GTG AGG     672
Arg Ser Leu Pro Gln Ala Leu Tyr Xaa Gly Thr Pro Ala Trp Val Arg
    210                 215                 220

GGG CCT GGA GGT CCC GGG GAA CCA CGG ATG TCT GTG GCC CAA TCC AAG     720
Gly Pro Gly Gly Pro Gly Glu Pro Arg Met Ser Val Ala Gln Ser Lys
225                 230                 235                 240

CTG CCT GGC CCG TGG GTC TTA TTT ACG TCG CAT CAT GTT TGG TGT GGG     768
Leu Pro Gly Pro Trp Val Leu Phe Thr Ser His His Val Trp Cys Gly
                245                 250                 255

CGA TGG ACA GTG TGC ACA TGC CAT GAT GGT GCC ATA CAA GCC TTT GAC     816
Arg Trp Thr Val Cys Thr Cys His Asp Gly Ala Ile Gln Ala Phe Asp
            260                 265                 270

AGC TTG CAG CCA TCA GTT CTG CAG GAC CAG ACT GCT GGG TGC TGT TTC     864
Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys Cys Phe
        275                 280                 285

CCG CGG GCA AGG CAC GAG TGG CCT GAG AAG AGC TGG AGG CAG AAA CAG     912
Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln
    290                 295                 300

TCC TTG TTT TGT CCT AAC GCC CAA GGT GTC CTG GCT GTA TGC ACT CAC     960
Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His
305                 310                 315                 320

TGC CCT GGC TCT CCA GGC CCT GCT CTG ATT AGG AAC ATG AAC CGT GGA    1008
Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly
                325                 330                 335

CGA CAC AGC TG                                                     1019
Arg His Ser
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 339 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Cys Gly Met Trp Arg Thr Lys His Gly Gly Leu Gln Pro Ala Arg Pro
 1               5                  10                  15

Ser Pro Gly Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala
            20                  25                  30

Ala Glu Ala Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu
        35                  40                  45

Tyr Val Ala Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly
    50                  55                  60

Pro Gly Gly Cys Val Arg Ser Arg Cys Arg Arg Pro Leu Arg Arg Phe
65                  70                  75                  80

Phe Ala Arg Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly
                85                  90                  95

Cys Glu Gly Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro
            100                 105                 110

Ala Cys Ala Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu
        115                 120                 125

Pro Leu Glu Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu
    130                 135                 140

Ala Phe Gln Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys
145                 150                 155                 160

Pro Glu Glu Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile
                165                 170                 175

Gly Thr Val Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val
            180                 185                 190

Ala Pro Trp Cys Gly Cys Ala Ala Xaa Trp Lys Pro Ala Arg Arg Met
        195                 200                 205

Arg Ser Leu Pro Gln Ala Leu Tyr Xaa Gly Thr Pro Ala Trp Val Arg
    210                 215                 220

Gly Pro Gly Gly Pro Gly Glu Pro Arg Met Ser Val Ala Gln Ser Lys
225                 230                 235                 240
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Leu Pro Gly Pro Trp Val Leu Phe Thr Ser His His Val Trp Cys Gly
                245                 250                 255

Arg Trp Thr Val Cys Thr Cys His Asp Gly Ala Ile Gln Ala Phe Asp
            260                 265                 270

Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys Cys Phe
        275                 280                 285

Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln
    290                 295                 300

Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His
305                 310                 315                 320

Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly
                325                 330                 335

Arg His Ser
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1017 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1017

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TGT GGA ATG TGG AGA ACC AAG CAC GGA GGA CTG CAG CCT GCC CGC CCT      48
Cys Gly Met Trp Arg Thr Lys His Gly Gly Leu Gln Pro Ala Arg Pro
  1               5                  10                  15

TCA CCA GGG TCT GCG AGC TTT ACC GAC GGG AAT CGC TGC GTG GAC GCG      96
Ser Pro Gly Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala
            20                  25                  30

GCC GAG GCG TGT ACA GCA GAC GAG CGG TGC CAG CAG CTG CGC TCT GAG     144
Ala Glu Ala Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu
        35                  40                  45

TAC GTG GCA CGA TGC CTG GGC CGG GCA GCG CCC GGG GGC AGG CCG GGA     192
Tyr Val Ala Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly
    50                  55                  60
```

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CCC GGG GGC TGC GTG CGC TCC CGC TGC CGC CGA CCC CTG CGC CGC TTC     240
Pro Gly Gly Cys Val Arg Ser Arg Cys Arg Arg Pro Leu Arg Arg Phe
 65                  70                  75                  80

TTC GCG CGT GGG CCT CCG GCG CTC ACG CAT GCG CTG CTC TTC TGC GGC     288
Phe Ala Arg Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly
                 85                  90                  95

TGC GAA GGC TCC GCG TGC GCC GAG CGC CGG CGC CAG ACT TTC GCG CCC     336
Cys Glu Gly Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro
            100                 105                 110

GCC TGC GCG TTC TCC GGC CCG GGG TTG GTG CCG CCC TCT TGC CTG GAG     384
Ala Cys Ala Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu
        115                 120                 125

CCC CTG GAG CGC TGC GAG CGC AGC CGC CTG TGC CGG CCC CGT CTC CTT     432
Pro Leu Glu Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu
    130                 135                 140

GCC TTC CAG GCC TCA TGC GCT CCC GCG CCC GGC TCC CGC GAC CGC TGC     480
Ala Phe Gln Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys
145                 150                 155                 160

CCG GAG GAG GGG GGC CCG CGT TGT CTG CGC GTC TAC GCA GGC CTC ATA     528
Pro Glu Glu Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile
                165                 170                 175

GGC ACC GTG GTC ACC CCC AAC TAC CTG GAC AAC GTG AGC GCG CGC GTT     576
Gly Thr Val Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val
            180                 185                 190

GCG CCC TGG TGC GGC TGT GCG GCC ANG TGG AAA CCG GCG CGA AGA ATG     624
Ala Pro Trp Cys Gly Cys Ala Ala Xaa Trp Lys Pro Ala Arg Arg Met
        195                 200                 205

CGA AGC CTT CCG CAA GCT CTT TAC AAN GGA ACC CCT GCT TGG GTG AGG     672
Arg Ser Leu Pro Gln Ala Leu Tyr Xaa Gly Thr Pro Ala Trp Val Arg
    210                 215                 220

GGG CCT GGA GGT CCC GGG GAA CCA CGG ATG TCT GTG GCC CAA TCC AAG     720
Gly Pro Gly Gly Pro Gly Glu Pro Arg Met Ser Val Ala Gln Ser Lys
225                 230                 235                 240

CTG CCT GGC CCG TGG GTC TTA TTT ACG TCG CAT CAT GTT TGG TGT GGG     768
Leu Pro Gly Pro Trp Val Leu Phe Thr Ser His His Val Trp Cys Gly
                245                 250                 255

CGA TGG ACA GTG TGC ACA TGC CAT GAT GGT GCC ATA CAA GCC TTT GAC     816
Arg Trp Thr Val Cys Thr Cys His Asp Gly Ala Ile Gln Ala Phe Asp
            260                 265                 270
```

```
AGC TTG CAG CCA TCA GTT CTG CAG GAC CAG ACT GCT GGG TGC TGT TTC    864
Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys Cys Phe
        275                 280                 285

CCG CGG GCA AGG CAC GAG TGG CCT GAG AAG AGC TGG AGG CAG AAA CAG    912
Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln
    290                 295                 300

TCC TTG TTT TGT CCT AAC GCC CAA GGT GTC CTG GCT GTA TGC ACT CAC    960
Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His
305                 310                 315                 320

TGC CCT GGC TCT CCA GGC CCT GCT CTG ATT AGG AAC ATG AAC CGT GGA   1008
Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly
                325                 330                 335

CGA CAC AGC                                                      1017
Arg His Ser
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06631

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/705; C12N 15/12, 15/63, 15/74, 15/79; C07H 21/00
US CL :530/350; 536/23.5, 24.31; 435/69.1, 252.3, 254.11, 320.1, 325
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.5, 24.31; 435/69.1, 252.3, 254.11, 320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Medline, WPI
search terms: Glial Derived Neurotrophic Factor Receptor, GDNF Receptor, DNA, cloning

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JING et al. GDNF-Induced Activation of the Ret Protein Tyrosine Kine is mediated by GDNFR-α, a Novel Receptor for GDNF. Cell. 28 June 1996, Vol. 85, pages 1113-1124.	1-9, 11, 14, 17
A	JING et al. GFRα-2 and GFRα-3 are Two New Receptors for Ligands of the GDNF Family. The Journal of Biological Chemistry. 26 December 1997, Vol. 272, pages 33111-33117.	1-9, 11, 14, 17
A	BALOH et al. TrnR2, a Novel Receptor that Mediates Neurturin and GDNF Signaling through Ret. Neuron, May 1997, Vol. 18, pages 793-802.	1-9, 11, 14, 17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of th actual completion of the international search

16 JUNE 1999

Date of mailing of the international search report

16 JUL 1999

Name and mailing address f the ISA/US
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Sally P. Teng

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F rm PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06631

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NOMOTO et al. Molecular Cloning and Expression Analysis of GFRα-3, a Novel cDNA Related to GDNFRα and NTNRα. Biochemical and Biophysical Research Communications. 27 March 1998, Vol. 244, pages 849-853.	1-9, 11, 14, 17
X, P ----- Y, P	EMBL/GenBank Database, Accession No. AU035938, SASAKI, Z., 'Construction of Mouse Full Length-Enriched cDNA Libraries,' abstract, Katsuyuki Hashimoto, National Institute of Infectious Diseases, Division of Genetic Resources, October 1998, see whole document.	1 ---------- 2-9, 11, 14, 17

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06631

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9, 11, 14, and 17

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied th payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06631

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-9, 11, 14, and 17, drawn to nucleic acid and the encoded polypeptide having SEQ ID NO: 2.
Group II, claim 10, drawn to antibodies.
Group III, claims 12 and 13, drawn to a method of detecting the presence of a polypeptide having SEQ ID NO: 2.
Group IV, claims 15 and 16, drawn to a method of detecting the presence of the nucleic acid molecule encoding SEQ ID NO: 2.
Group V, claims 18 and 19, drawn to a method of identifying a compound that binds a protein having SEQ ID NO: 2.
Group VI, claim 20, drawn to a method of modulating the activity of a protein having SEQ ID NO: 2.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of Group I is the nucleic acid sequence encoding the polypeptide having SEQ ID NO: 2. The special technical feature of Group II is the antibodies that bind to the polypeptide but does not have the amino acid sequence of the polypeptide. The special technical feature of Group III is a method of detecting the presence of a polypeptide using a compound that bind to the polypeptide. The special technical feature of Group IV is a method of detecting the presence of a nucleic acid molecule using a nucleic acid encoding SEQ ID NO: 2. The special technical feature of Group V is a method of identifying a compound that binds a protein having SEQ ID NO: 2 using the polypeptide and a test compound. The special technical feature of Group VI is a method of modulating the activity of a protein using a cell expressing the protein and a compound that binds the protein. The special technical feature of each group is not the same or does not correspond to the special technical feature of any other group because the products of Groups I and II are structurally and functionally distinct and the methods of Groups III-VI require different method steps and starting reagents for achieving different goals. The groups are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/705, C12N 15/12, 15/63, 15/74, 15/79, C07H 21/00

A1

(11) International Publication Number: WO 99/50298

(43) International Publication Date: 7 October 1999 (07.10.99)

(21) International Application Number: PCT/US99/06631

(22) International Filing Date: 25 March 1999 (25.03.99)

(30) Priority Data:
60/080,070 31 March 1998 (31.03.98) US

(71) Applicant: MILLENNIUM PHARMACEUTICALS, INC. [US/US]; 640 Memorial Drive, Cambridge, MA 02139 (US).

(72) Inventor: MOORE, Karen, J.; 34 Chandler Street, Maynard, MA 01754 (US).

(74) Agents: MANDRAGOURAS, Amy, E.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US) et al.

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: GFRα–X, A NOVEL GLIAL–DERIVED NEUROTROPHIC FACTOR RECEPTOR AND USES THEREFOR

(57) Abstract

The invention provides isolated nucleic acids molecules that encode new members of the GFRα family of protein, designated *GFRα–X* for GDNF Family Receptor Alpha–X nucleic acid molecules. This family of proteins bind neurotrophic factors and mediate signals involved in the regulation of neural cell functions. The invention also provides antisense nucleic acid molecules, expression vectors containing *GFRα–X* nucleic acid molecules, host cells into which the expression vectors have been introduced, and non–human transgenic animals in which a *GFRα–X* gene has been introduced or disrupted. The invention still further provides isolated GFRα–X polypeptides, fusion proteins, antigenic peptides, and anti–GFRα–X antibodies. Diagnostic, screening, and therapeutic methods utilizing compositions of the invention are also provided.

*(Referred to in PCT Gazette No. 47/1999, Section II)

BNSDOCID: <WO___9950298A1_IA>

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CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

GFRα-X, A NOVEL GLIAL-DERIVED NEUROTROPHIC FACTOR RECEPTOR AND USES THEREFOR

Background of the Invention

Two recently described growth factors, glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN), have been found to be approximately 20% similar to other TGF-β family members. This similarity is based primarily on the seven cysteine residues found in the same relative spacing across the entire TGF-β family of growth factors. Mature GDNF and mature NTN, however, have a high similarity (approximately 42%) to one another relative to the other TGF-β family members. Thus, these neurotrophic factors represent a subfamily of new growth factors within the TGF-β superfamily of growth factors and may be more closely related than their homology suggests. Due to their potent neurotrophic effects on a variety of neural cell types, moreover, GDNF and NTN comprise a subfamily of neurotrophic factors. As GDNF was cloned several years earlier than NTN (Lin et al. ((1993) *Science* 260(5111):1130-1132; Kotzbauer, P.T. et al. (1996) *Nature* 384:467-470), the effects of GDNF on various types of neural cells have been better characterized than the effects of NTN on neural cells.

GDNF was first characterized as promoting survival of cultured dopaminergic neurons of the substantia nigra (Lin et al. ((1993) *Science* 260(5111):1130-1132). GDNF was subsequently found to have potent effects on a wide range of additional neural populations. For example, GDNF was found to promote the survival of primary cultures of spinal motorneurons (Henderson, C.E. et al. (1994) *Science* 266:1062-1064) and mutated motorneurons *in vivo* (Li, L.X. et al. (1995) *PNAS* 92:9771-9775; Oppenheim, R. et al. (1995) *Nature* 373:344-346; Yan, Q. et al. (1995) *Nature* 373:341-344). Additionally, GDNF has been observed to have pronounced effects on cultures of dissociated neurons from various chick peripheral ganglia--sympathetic, sensory, and enteroceptive (Bujbello, A. et al. (1995) *Neuron* 15:821-828; Ebendal, T. et al. (1995) *Cell Growth & Diff.* 7:1081-1086;Trupp, M. et al. (1995) *J.Cell. Biol.* 130:137-148). GDNF has also been shown to promote the survival and morphologic differentiation of

primary cultures of Purkinje cells (Mount, H.T.J. et al. (1995) *PNAS* 92:9092-9096). Still other functions of GDNF include the ability to prevent degeneration and promote the phenotype of brain noradrenergic neurons *in vivo* (Arenas, E. et al. (1995) *Neuron* 15:1465-1473), to sustain axotomized basal forebrain cholinergic neurons *in vivo* (Williams, L.R. et al. (1996) *J. Pharmacol. Exp. Ther.* 277:1140-1151), and to inhibit kainic acid mediated seizures in rat (Martin, D. et al. (1995) *Brain Res.* 683:172-178). In addition to these effects in the adult central nervous systems, GDNF plays a critical role as a morphogen in the developing excretory and enteric nervous systems. This role is evident in the fact that mice defective in GDNF expression display complete renal agenesis and lack of enteric neurons (Moore, M.W. et al. (1996) *Nature* 382:76-79; Pichel, J.G. et al. (1996) *Nature* 382:73-76; Sanchez, M.P. et al. (1996) *Nature* 382:70-73).

NTN has been characterized as promoting survival of nodose ganglia sensory neurons, dorsal root ganglia sensory neurons, and superior cervical ganglia sympathetic neurons *in vitro* (Kotzbauer, P.T. et al. (1996) *Nature* 384:467-470). NTN's effect on other neural cell types has not yet been determined.

GDNF and NTN signal cells, e.g., neural cells and other cell types, in many instances, via a multicomponent receptor system formed by a glycosyl-phosphatidylinositol (GPI)-linked ligand binding subunit (the "α" subunit) and the tyrosine kinase receptor RET as a signaling ("β") subunit. Jing, S. et al. (1996) *Cell* 85:1113-1124; Treanor, J.J.S. et al. (1996) *Nature* 382:80-83. Binding of these neurotrophic factors to the α subunit promotes formation of a physical complex between the α and β subunits, thereby inducing tyrosine phosphorylation of the β subunit. Tyrosine phosphorylation of the β subunit results in transmission of the GDNF/NTN signal to the interior of the cell.

Several genes encoding α subunits of this GDNF/NTN receptor complex have been cloned and characterized. The first member of this receptor family, GDNF receptor-α (GDNFR-α), which has been renamed GFRα-1 for ***G***DNF ***F***amily ***R***eceptor ***A***lpha-***1*** by the GFRα Nomenclature Committee (GFRα Nomenclature Committee (1997) *Nature* 19:485) has been shown to bind to GDNF and to mediate binding and

activation of the RET receptor tyrosine kinase (Jing, S.Q. et al. (1996) *Cell* 85:1113-1124; Treanor, J.J.S. et al. (1996) *Nature* 382:80-83). The second member of the receptor family, alternatively named TrnR-2, NTNR-α, RETL2, and GDNFR-β, which has been renamed GFRα-2 by the GFRα Nomenclature Committee (GFRα Nomenclature Committee (1997) *Nature* 19:485), has been shown to bind NTN and to mediate activation of RET by both NTN and GDNF (Baloh, R.H. et al. (1997) *Neuron* 18:793-802; Bujbello, A. et al. (1997) *Nature* 387:721-724).

A third member of the receptor family, GRFα-3, has been described at recent scientific conferences.

Summary of the Invention

The invention is based on the discovery of nucleic acid molecules that encode a fourth member of the Glial Derived Neurotrophic Factor-Alpha Family of Receptors, hereinafter the *GFRα-X* cDNA, as well as the GFRα-X protein. The first member of the *GFRα-X* subfamily was identified, as described herein, in a positional cloning process in which the mouse mahogany locus was being sequenced to identify genes involved in obesity. Nucleic acid molecules encoding the GFRα-X proteins are referred to herein as *GFRα-X* nucleic acid molecules.

The GFRα-X proteins of the present invention bind to neurotrophic factors, such as GDNF and/or NTN, and mediate signals within cells expressing the GFRα-X protein. Typically, the GFRα-X protein transmits a signal to the interior of the cell by activation of the RET protein tyrosine kinase signalling pathway. Neurotrophic factors promote survival and function of neural cells of both the central and peripheral nervous systems. Thus, modulation of the activity of a molecule involved in transmitting a neurotrophic factor signal to a cell (e.g., GFRα-X) results in modulation of the neurotrophic factor initiated cell function. Consequently, modulation of GFRα-X function can be used to modulate neurotrophic factor action/activity and thereby treat disorders associated with such functions (or lack thereof).

In addition, GFRα proteins are expressed in a variety of cell lineages in the brain (for example Lateral septal neurons, Septohypothalamic neurons, paraventricular thalamic neurons (anterior), superchiasmatic neurons, anterior cortical amygdaloid neurons, piriform cortex, paracentral thalamic neurons, lateral habenular neurons, paraventricular hypothalamic neurons (PVN), amygdaloid nucleus area, arcuate neurons, and ventromedial hypothalamic neurons (VMH)) and during embryogenesis, including, for example, cells of the midbrain, motorneurons, cells of the enteric nervous system, embryonic smooth and striated muscles around the enteric nervous system in the esophagus, gut and stomach, developing nephrons and cells of the pancreatic primordium. Thus, modulators of GFRα-X can be used to modulate development of these tissues to thereby treat disorders associated with abnormal or aberrant development of these various tissues.

Accordingly, one aspect of the invention provides isolated nucleic acid molecules (e.g., cDNAs) comprising a nucleotide sequence encoding a GFRα-X protein or a fragment thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of GFRα-X-encoding nucleic acid (e.g., mRNA). In particularly preferred embodiments, the isolated nucleic acid molecule comprises a nucleic acid molecule which encodes the amino acid sequence of SEQ ID NO:2, such as the nucleotide sequence of SEQ ID NO:1. In other particularly preferred embodiments, the isolated nucleic acid molecule of the invention comprises a nucleotide sequence which hybridizes to or is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence shown in SEQ ID NO:1.

In another embodiment, the isolated nucleic acid molecule encodes a protein or portion thereof wherein the protein or portion thereof includes an amino acid sequence which is sufficiently homologous to a protein comprising an amino acid sequence of SEQ ID NO:2, such that the protein or portion thereof maintains a GFRα-X activity. Preferably, the protein or portion thereof encoded by the nucleic acid molecule maintains the ability to bind to a neurotrophic factor and modulate a cellular response. In one embodiment, the protein encoded by the nucleic acid molecule is at least about

30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the amino acid sequence of SEQ ID NO:2. In another preferred embodiment, the protein is a full length protein which is substantially homologous to the entire amino acid sequence of SEQ ID NO:2, such the naturally occurring full length protein, and all allelic variants and splice variants of human and murine GFRα-X.

In another embodiment, the isolated nucleic acid molecule is at least 15 nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1. Preferably, the isolated nucleic acid molecule corresponds to a naturally occurring nucleic acid molecule. More preferably, the isolated nucleic acid encodes naturally-occurring alleles and splice variants of human GFRα-X. Moreover, given the disclosure herein of a GFRα-X-encoding cDNA sequence (e.g., SEQ ID NO:1), antisense nucleic acid molecules (i.e., molecules which are complementary to the coding strand of the GFRα-X cDNA sequence) are also provided by the invention.

Another aspect of the invention pertains to vectors, e.g., recombinant expression vectors, containing the nucleic acid molecules of the invention and host cells into which such vectors have been introduced. In one embodiment, such a host cell is used to produce GFRα-X protein by culturing the host cell in a suitable medium. If desired, the GFRα-X protein can then be isolated from the host cell.

Yet another aspect of the invention pertains to transgenic non-human animals in which a *GFRα-X* gene has been introduced or altered. In one embodiment, the genome of the non-human animal has been altered by introduction of a nucleic acid molecule of the invention encoding GFRα-X as a transgene. In another embodiment, an endogenous *GFRα-X* gene within the genome of the non-human animal has been altered, e.g., functionally disrupted, by homologous recombination.

Still another aspect of the invention pertains to an isolated GFRα-X protein or a portion, e.g., a biologically active portion, thereof. In a preferred embodiment, the isolated GFRα-X protein or portion thereof can bind a neurotrophic factor and stimulate a response in a neurotrophic factor responsive cell.

The invention also provides an isolated preparation of a GFRα-X protein. In preferred embodiments, the GFRα-X protein comprises the amino acid sequence of SEQ ID NO:2. In another preferred embodiment, the invention pertains to an isolated full length protein which is substantially homologous to the entire amino acid sequence of SEQ ID NO:2 (containing additional 5' sequence). In yet another embodiment, the protein is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the entire amino acid sequence of SEQ ID NO:2. In other embodiments, the isolated GFRα-X protein comprises an amino acid sequence which is at least about 60-70% or more homologous to the amino acid sequence of SEQ ID NO:2 and has one or more of the following activities: 1) it can interact with (e.g., bind to) a neurotrophic factor, e.g., GDNF and/or NTN; 2) it can interact with (e.g., bind to) a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; 3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and 4) it can bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell, e.g., a neural cell, a cell of the developing digestive tract, or a cell of its associated nervous system innervation, to, for example, beneficially affect the cell. Alternatively, the isolated GFRα-X protein can comprise an amino acid sequence which is encoded by a nucleotide sequence which hybridizes, e.g., hybridizes under stringent conditions, or is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence of SEQ ID NO:1. It is also preferred that the preferred forms of GFRα-X also have one or more of the GFRα-X activities described herein.

The GFRα-X protein (or polypeptide) or a biologically active portion thereof can be operatively linked to a non-GFRα-X polypeptide to form a fusion protein.

The GFRα-X protein of the invention, or portions or fragments thereof, can be used to prepare anti-GFRα-X antibodies. Accordingly, the invention also provides an antigenic peptide of GFRα-X which comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of GFRα-X such that an antibody raised against the peptide forms a specific immune complex with GFRα-X. Preferably, the antigenic peptide comprises at least 10 amino acid residues,

more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues and has a high antigenicity index as shown in Figure 2. The invention further provides an antibody that specifically binds GFRα-X. In one embodiment, the antibody is monoclonal. In another embodiment, the antibody is coupled to a detectable substance. In yet another embodiment, the antibody is incorporated into a pharmaceutical composition comprising the antibody and a pharmaceutically acceptable carrier.

Another aspect of the invention pertains to methods for modulating a GFRα-X mediated cell activity, e.g., function, proliferation or differentiation. Such methods include contacting the cell with an agent which modulates a GFRα-X protein activity or *GFRα-X* nucleic acid expression such that a cell associated activity is altered relative to a cell associated activity (e.g., the same cell associated activity) of the cell in the absence of the agent. In a preferred embodiment, the cell is capable of responding to a neurotrophic factor through a signaling pathway involving a GFRα-X protein. The agent which modulates GFRα-X activity can be an agonist agent, an agent which stimulates GFRα-X protein activity or *GFRα-X* nucleic acid expression; or an antagonist agent, an agent which inhibits GFRα-X protein activity or *GFRα-X* nucleic acid expression. Examples of agents which stimulate GFRα-X protein activity or *GFRα-X* nucleic acid expression include small molecules and nucleic acids encoding GFRα-X that have been introduced into the cell. Examples of agents which inhibit GFRα-X activity or expression include small molecules, antisense *GFRα-X* nucleic acid molecules, and antibodies that specifically bind to GFRα-X. In a preferred embodiment, the cell is present within a subject and the agent is administered to the subject.

The present invention also pertains to methods for treating subjects having disorders mediated by abnormal GFRα-X activity/expression. For example, the invention pertains to methods for treating a subject having a disorder characterized by aberrant GFRα-X protein activity or nucleic acid expression such as a neurological disorder, e.g., a central nervous system disorder, e.g., Parkinson's disease, or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development. These methods

include administering to the subject a GFRα-X modulator (e.g., a small molecule) such that treatment of the subject occurs.

In another embodiment, the invention pertains to methods for treating a subject having a neurological disorder, e.g., a central nervous system disorder, e.g., Parkinson's disease, or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development, comprising administering to the subject a GFRα-X modulator such that treatment occurs.

In other embodiments, the invention pertains to methods for treating a subject having a neurological disorder, e.g., a central nervous system disorder, e.g., Parkinson's disease or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development, comprising administering to the subject a GFRα-X protein or portion thereof such that treatment occurs. Neurological disorders and disorders associated with abnormal or aberrant cell, e.g., neural cell, development can also be treated according to the invention by administering to the subject having the disorder a nucleic acid encoding a GFRα-X protein or portion thereof such that treatment occurs.

The invention also pertains to methods for detecting genetic mutations in a *GFR α-X* gene, thereby determining if a subject with the mutated gene is at risk for (or is predisposed to have) a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity, e.g., a central nervous system disorder, e.g., Parkinson's disease or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic mutation characterized by an alteration affecting the integrity of a gene encoding a GFRα-X protein, or the misexpression of the *GFRα-X* gene.

Another aspect of the invention pertains to methods for detecting the presence of GFRα-X, or fragment thereof, in a biological sample. In a preferred embodiment, the methods involve contacting a biological sample (e.g., a neural cell sample) with a compound or an agent capable of detecting GFRα-X protein or GFRα-X encoding mRNA such that the presence of GFRα-X is detected in the biological sample. The compound or agent can be, for example, a labeled or labelable nucleic acid probe

capable of hybridizing to GFRα-X encoding mRNA or a labeled or labelable antibody capable of binding to GFRα-X protein. The invention further provides methods for diagnosis of a subject with, for example, a central nervous system disorder, e.g., Parkinson's disease, or a disorder associated with abnormal or aberrant cell, e.g., neural cell, development, based on detection of GFRα-X protein or mRNA. In one embodiment, the method involves contacting a cell, tissue, or fluid sample (e.g., a neural cell sample) from the subject with an agent capable of detecting GFRα-X protein or mRNA, determining the amount of GFRα-X protein or mRNA expressed in the sample, comparing the amount of GFRα-X protein or mRNA expressed in the sample to a control sample and forming a diagnosis based on the amount of GFRα-X protein or mRNA expressed in the sample as compared to the control sample. Preferably, the sample is a neural cell sample. Kits for detecting GFRα-X, or fragments thereof, in a biological sample are also within the scope of the invention.

Still another aspect of the invention pertains to methods, e.g., screening assays, for identifying a compound for treating a disorder characterized by aberrant *GFRα-X* nucleic acid expression or protein activity, e.g., a central nervous system disorder, e.g., Parkinson's disease or a disorder associated with abnormal or aberrant cell, i.e., neural cell, development. These methods typically include assaying the ability of the compound or agent to modulate the expression of the *GFRα-X* gene or the activity of the GFRα-X protein thereby identifying a compound for treating a disorder characterized by aberrant *GFRα-X* nucleic acid expression or protein activity. In a preferred embodiment, the method involves contacting a biological sample obtained from a subject having the disorder with the compound or agent, determining the amount of GFR α-X protein expressed and/or measuring the activity of the GFRα-X protein in the biological sample, comparing the amount of GFRα-X protein expressed in the biological sample and/or the measurable GFRα-X biological activity in the cell to that of a control sample. An alteration in the amount of GFRα-X protein expression or GFRα-X activity in the cell exposed to the compound or agent in comparison to the control is indicative of a modulation of GFRα-X expression and/or GFRα-X activity.

The invention also pertains to methods for identifying a compound or agent which interacts with (e.g., binds to) a GFRα-X protein. These methods can include the steps of contacting the GFRα-X protein, a fragment thereof, or a cell expressing GFRα-X, with the compound or agent under conditions which allow binding of the compound to the GFRα-X protein to form a complex and detecting the formation of a complex of the GFRα-X protein and the compound in which the ability of the compound to bind to the GFRα-X protein is indicated by the presence of the compound in the complex.

The invention further pertains to methods for identifying a compound or agent which modulates, e.g., stimulates or inhibits, the interaction of the GFRα-X protein with a target molecule, e.g., GDNF, NTN, a complex of GDNF and NTN, or the tyrosine kinase receptor RET. In these methods, the GFRα-X protein is contacted, in the presence of the compound or agent, with the target molecule under conditions which allow binding of the target molecule to the GFRα-X protein to form a complex. An alteration, e.g., an increase or decrease, in complex formation between the GFRα-X protein and the target molecule as compared to the amount of complex formed in the absence of the compound or agent is indicative of the ability of the compound or agent to modulate the interaction of the GFRα-X protein with a target molecule.

Brief Description of the Drawing

Figure 1 depicts the mouse GFRα-X nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence.

Figure 2 depicts a structural analysis of the mouse GFRα-X protein.

Figure 3 provides an alignment of the amino acid sequence of members of the GRFα family of protein.

Detailed Description of the Invention

The present invention is based on the discovery of novel molecules, referred to herein as *GFRα-X* nucleic acid molecules and GFRα-X proteins, which function in neurotrophic factor signaling pathways. As used herein, "a neurotrophic factor" refers to a protein that modulates a biological activity of a cell, particularly a neuronal cell,

through a neurotrophic factor signaling pathway, such as the RET signaling pathway. Examples of biological activities include, but are not limited to, neural cell survival and/or neural cell function. Examples of such neurotrophic factors include GDNF and NTN.

As used herein, "RET signaling pathway" includes a cell, e.g., neural cell, signaling pathway which involves the tyrosine kinase receptor RET. An example of such a pathway includes the GDNF or NTN (neurotrophic factor) signaling pathway.

As used herein, "a neurotrophic factor responsive cell" includes a cell which has a biological activity that can be modulated (e.g., stimulated or inhibited) by a neurotrophic factor. Examples of such functions include mobilization of intracellular molecules which participate in a signal transduction pathway, production or secretion of molecules, alteration in the structure of a cellular component, cell proliferation, cell migration, cell differentiation, and cell survival. Cells responsive to neurotrophic factors preferably express a neurotrophic factor receptor, e.g., a GFRα receptor, such as GFRα-X, and/or a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET. Examples of neurotrophic factor responsive cells include neural cells, e.g., cells of the central nervous system and peripheral nervous system cells (e.g., sympathetic and parasympathetic neurons), cells of the enteric nervous system, embryonic smooth and striated muscles around the enteric nervous system in the esophagus, gut and stomach, developing nephrons, and cells of the pancreatic primordium.

Depending on the type of cell, the response elicited by neurotrophic factors is different. For example, in neural cells, neurotrophic factors regulate neural survival and neural function. Abnormal or aberrant activity of proteins involved in the neurotrophic signaling pathway can lead to a variety of neurological disorders, e.g., central nervous system disorders. For example, abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the thalamus (e.g., the reticular thalamic nucleus, the zona certa, the anteromedial and dorsal thalamic nucleus, the lateral habenular nucleus, and the medial habenular nucleus) can lead to sensory disorders. Sensory disorders are disorders which detrimentally affect normal sensory function. Examples of such sensory disorders include Dejerine-Roussy syndrome, contralateral

anesthesia, and dense hypesthesia. Abnormal or aberrant activity in cells of the peripheral or enteric nervous system can lead to eating disorders.

Abnormal or aberrant activity of a GFRα-X (or abnormal or aberrant nucleic acid expression of the nucleic acid encoding the protein)in a neurotrophic signaling factor pathway in the midbrain or mesencephalon (e.g., the substantia nigra compacta and scattered cells of SN reticulata, the ventral segmental area, the interpenduncular nucleus, the supramammilary nucleus, the red nucleus, and the dorsal raphe nucleus) can lead to motor disorders. Motor disorders are disorders which detrimentally affect normal motor functions. Examples of such motor disorders include ataxia, facial infarction, tremors, tics, athetosis, amyotrophic lateral sclerosis (ALS), and Parkinson's disease.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the cerebellum (e.g., the Purkinje layer, the molecular layer, and the deep cerebellar nuclei) can also lead to motor disorders. Examples of such motor disorders include loss of equilibrium and multiple sclerosis.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the pons (e.g., the pontine reticular nucleus, the pontine nucleus, the motor trigeminal nucleus, the inferior olive nuclei, the locus coeruleus, the dorsal cochlear nucleus, the facial nucleus, the vestibular nucleus, and the hypoglossal nucleus) can lead to motor disorders. Examples of such motor disorders include facial palsy, and limb ataxia.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the olfactory system (e.g., the olfactory tubercule, the internal granular layer of olfactory bulb, the external plexiform layer of olfactory bulb, the glomerular layer, and the olfactory nerve layer) can lead to sensory disorders. Sensory disorders are disorders which detrimentally affect normal sensory function. An example of such a sensory disorder includes the loss of olfaction functionality.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor in a signaling pathway in the neocortex (also known as the neopallium or isocortex) (e.g., the hippocampus) can lead to cognitive disorders. Cognitive disorders are disorders which

detrimentally affect normal cognitive functions. An example of such a cognitive disorder is Alzheimer's disease.

Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in the amygdala can lead to motor disorders. Examples of such motor disorders include athetosis, dystoia, and tremors.

In addition, neurotrophic factors such as GDNF and NTN and the interaction with GFRs also promote proper development of a variety of cell types. For example, neurotrophic factor/receptor interactions promote development and function of certain peripheral organs and cells of their associated nervous system innervation. Abnormal or aberrant activity of GFRα-X in a neurotrophic factor signaling pathway in these peripheral organs (e.g., kidneys, testis, intestine, stomach, heart, lung and skin) can lead to disorders associated with cellular development of cells of these organs. An example of a disorder associated with development of the enteric nervous system is Hirschsprung's disease. Examples of disorders associated with development of the kidneys include kidney dysfunction, renal agenesis, and severe dysgenesis.

A murine *GFRα-X* nucleic acid molecule was identified from a positional cloning process in which the mouse mahogany locus was being sequenced to identify genes involved in obesity (described in detail in Example 1). During sequencing of a larger genomic region, an open reading frame was identified that encoded a protein that showed sequence homology to GFRα-1. Probes were generated based on portions of the genomic sequence and cDNA libraries were screened. Nucleotide sequences were determined and assembled and various methods such as RACE and genomic sequence analysis were used to extend the 5' sequence. The nucleotide sequence of the isolated mouse *GFRα-X* cDNA and the predicted amino acid sequence of the mouse GFRα-X protein are shown in Figure 1 and in SEQ ID NOs:1 and 2, respectively. A plasmid containing the nucleotide sequence encoding mouse GFRα-X was deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, on _____ and assigned Accession Number __. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was

made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

The murine *GFRα-X* nucleic acid molecule is approximately 1019 nucleotides in length, and encodes a protein that is approximately 339 amino acid residues in length. This clone is likely to be missing several nucleotides (and amino acids) that are present at the 5' end of the naturally occurring cDNA. The GFRα-X protein is expressed at least in brain cells, particularly in Lateral septal neurons, Septohypothalamic neurons, paraventricular thalamic neurons (anterior), superchiasmatic neurons, anterior cortical amygdaloid neurons, piriform cortex, paracentral thalamic neurons, lateral habenular neurons, paraventricular hypothalamic neurons (PVN), amygdaloid nucleus area, arcuate neurons, and ventromedial hypothalamic neurons (VMH).

Various aspects of the invention are described in further detail in the following subsections:

I. Isolated Nucleic Acid Molecules

One aspect of the invention provides isolated nucleic acid molecules that encode GFRα-X proteins, particularly human or murine orthologues, biologically active portions thereof, as well as nucleic acid fragments sufficient for use as hybridization probes to identify GFRα-X-encoding nucleic acid molecules (e.g., GFRα-X encoding mRNA). As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule" includes nucleic acid molecules which are separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and

3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated *GFRα-X* nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived (e.g., a neural cell). Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, a homologue or orthologue or human or murine *GFRα-X* cDNA can be isolated from a cDNA library, such as a brain library, using all or portion of SEQ ID NO:1 as a hybridization probe and standard hybridization techniques (e.g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1 can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO:1. For example, mRNA can be isolated from neural cells (e.g., by the guanidinium-thiocyanate extraction procedure of Chirgwin et al. (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (e.g., Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon the nucleotide sequences shown in SEQ ID NO:1. A nucleic acid of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

Furthermore, oligonucleotides corresponding to a *GFRα-X* nucleotide sequence can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1. The sequence of SEQ ID NO:1 corresponds to the mouse *GFRα-X* cDNA.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1 or a portion of this nucleotide sequence. For example, a nucleic acid molecule which is complementary to the nucleotide sequence shown in SEQ ID NO:1 is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1 such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1, thereby forming a stable duplex.

In still another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence which is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence shown in SEQ ID NO:1. In an additional preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence which hybridizes, e.g., hybridizes under stringent conditions, to the nucleotide sequence shown in SEQ ID NO:1 or a portion of either of these nucleotide sequences. Preferably such nucleic acid molecules encode naturally occurring allelic variants of the mouse *GFRα-X* nucleic acid molecules disclosed herein or non-mouse orthologues, such as human GFRα-X.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the coding region of SEQ ID NO:1, for example a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of GFRα-X such as a ligand binding domain or signaling partner binding site of GFRα-X. The nucleotide sequence determined from the cloning of the *GFRα-X* gene from a mouse allows for the generation of probes and primers designed for use in identifying and/or cloning *GFRα-X* homologues in other cell types, e.g., from other tissues, as well as *GFRα-X* orthologues from other mammals such as humans. The probe/primer typically comprises

substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 40, 50 or 75 consecutive nucleotides of SEQ ID NO:1 sense, an anti-sense sequence of SEQ ID NO:1, or naturally occurring mutants thereof. Primers based on the nucleotide sequence in SEQ ID NO:1 can be used in PCR reactions to clone GFRα-X homologues. Probes based on the GFRα-X nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto, e.g., the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a GFRα-X protein, such as by measuring a level of a GFRα-X-encoding nucleic acid in a sample of cells from a subject, e.g., detecting GFRα-X encoding mRNA levels or determining whether a genomic *GFRα-X* gene has been mutated or deleted.

In one embodiment, the nucleic acid molecule of the invention encodes a protein or portion thereof which includes an amino acid sequence which is sufficiently homologous to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof maintains one or more of the activities possessed by GFRα-X. Examples of such homologous proteins include, but are not limited to, allelic variants of SEQ ID NO:2 and non-mouse orthologues (such as human GFRα-X) of SEQ ID NO:2. As used herein, the language "sufficiently homologous" refers to proteins or portions thereof which have amino acid sequences which include a minimum number of identical or equivalent (e.g., an amino acid residue which has a similar side chain as an amino acid residue in SEQ ID NO:2) amino acid residues to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof is able to bind a neurotrophic and modulate a response in a neurotrophic factor responsive cell. In another embodiment, the protein is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the entire amino acid sequence of SEQ ID NO:2.

Portions of proteins encoded by the *GFRα-X* nucleic acid molecule of the invention preferably possess one or more activities of the GFRα-X protein. As used herein, the term "biologically active portion of GFRα-X" is intended to include a portion, e.g., a domain/motif, of GFRα-X that has one or more of the following activities: 1) it can interact with (e.g., bind to) a neurotrophic factor, e.g., GDNF and/or NTN; 2) it can interact with (e.g., bind to) a tyrosine kinase receptor or other signaling partner, e.g., the tyrosine kinase receptor RET; 3) it can modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET; and 4) it can bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell, e.g., a neural cell, a cell of the developing digestive tract, or a cell of its associated nervous system innervation, to, for example, beneficially affect the cell. Direct binding assays as described herein, can be performed to determine the ability of a GFRα-X protein or biologically active portion thereof to interact with (e.g., bind to) a neurotrophic factor (e.g., GDNF and/or NTN or a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET).

The ability of GFRα-X proteins of the present invention to interact with a neurotrophic factor can be determined using the following assay. Plasmids including a nucleic acid molecule which encodes a fragment of the mouse GFRα-X protein either alone or as a chimeric fusion protein with, for example, an Ig constant region can be generated, as described in Sanicola et al. (1991) *Proc. Natl. Acad. Sci.* 94:6238-6243, by ligating a DNA fragment encoding the GFRα-X fragment to suitable vector sequences. The plasmids can be transfected into 293-EBNA cells and stable lines obtained by using hygromycin selection. The GFRα-X fragment or fusion proteins can be purified and then exposed to rhGDNF (Promega, Madison, WI). Complexes of GFRα-X and rhGDNF can then be identified.

The ability of GFRα-X protein or a biologically active portion thereof to interact with (e.g., bind to) a tyrosine kinase receptor (e.g., the tyrosine kinase receptor RET) can be determined using an assay similar to the assay described above for determining the ability of a GFRα-X protein or biologically active portion thereof to interact with (e.g., bind to) a neurotrophic factor (e.g., GDNF and/or NTN). In particular, the mouse GFRα

-X protein as described above can be exposed to proteins known to complex with members of the GFRα-X family of receptors. Complexes can be identified and detected using art known methods.

The ability of a fragment of a GFRα-X protein of the present invention to modulate the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET, can be determined using the following assay. As described in Treanor et al. (1996) *Nature* 382:80-83, the human neuroblastoma SK-N-SH and the mouse neuroblastoma Neuro-2a, cell lines that express endogenous c-*ret*, can be exposed to GDNF alone or to GDNF in combination with a soluble fragment of GFRα-X for 5 minutes, and the level of RET tyrosine phosphorylation can be determined. To determine whether induction of RET tyrosine phosphorylation is dependent on the presence of the GFRα-X fragment, Neuro-2a and SK-N-SH cells can be treated with PIPLC, and the response of RET to GDNF can be examined. A change in tyrosine kinase RET phosphorylation in the cell lines treated with GDNF in combination with soluble GFRα-X compared to cell lines treated with GDNF alone indicates that the GFR α-X protein is capable of modulating the activity of a tyrosine kinase receptor, e.g., the tyrosine kinase receptor RET.

In one embodiment, the biologically active portion of GFRα-X comprises the N-terminal domain of the GFRα-X protein. Figure 2 provides a structural analysis of the mouse GFRα-X protein. Additional domains can e identified by analyzing conserved residue in the GFRα family of proteins (Figure 3). Additional nucleic acid fragments encoding biologically active portions of GFRα-X can be prepared by isolating a portion of SEQ ID NO:1, expressing the encoded portion of GFRα-X protein or peptide (e.g., by recombinant expression *in vitro*) and assessing the activity of the encoded portion of GFRα-X protein or peptide.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1 (and portions thereof) due to degeneracy of the genetic code and thus encode the same GFRα-X protein as that encoded by the nucleotide sequence shown in SEQ ID NO:1. In such an embodiment, an isolated

nucleic acid molecule of the invention has a nucleotide sequence encoding a protein comprising an amino acid sequence shown in SEQ ID NO:2.

In addition to the GFRα-X nucleotide sequence shown in SEQ ID NO:1, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of GFRα-X may exist within a population. Such genetic polymorphism in the *GFRα-X* gene may exist among individuals within a population due to natural allelic variation producing both active variants and inactive variants. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a GFRα-X protein, preferably a mammalian GFRα-X protein. Such active natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the *GFRα-X* gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in GFRα-X that are the result of natural allelic variation are intended to be within the scope of the invention. Moreover, nucleic acid molecules encoding GFRα-X proteins from other species, and thus which have a nucleotide sequence which differs from the mouse sequence of SEQ ID NO:1, are intended to be within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and human homologues of the mouse *GFRα-X* cDNA of the invention can be isolated based on their homology to the mouse *GFRα-X* nucleic acid disclosed herein using the mouse cDNA, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 15 nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1. In other embodiments, the nucleic acid is at least 30, 50, 100, 250 or 500 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least about 65%, more preferably at least about 70%, and even more preferably at least about 75% or more homologous to each other typically remain hybridized to each other. Such stringent

conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1 corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g., encodes a natural protein) and is encoded by a common genetic locus. In one embodiment, the nucleic acid encodes a natural human GFRα-X.

In addition to naturally-occurring allelic variants of the GFRα-X sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by directed or random mutation into the nucleotide sequence of SEQ ID NO:1, thereby leading to changes in the amino acid sequence of the encoded GFRα-X protein, without altering the functional ability of the GFRα-X protein. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence of SEQ ID NO:1. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of GFRα-X (e.g., the sequence of SEQ ID NO:2) without altering the activity of GFRα-X, whereas an "essential" amino acid residue is required for GFRα-X activity. For example, conserved amino acid residues, e.g., hydrophobic amino acids, in the N-terminal domain of GFRα-X are most likely important for binding to a neurotrophic factor and are thus essential residues of GFRα-X. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved in the N-terminal hydrophobic domain) may not be essential for activity and thus are likely to be amenable to alteration without altering GFRα-X activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding GFRα-X proteins that contain changes in amino acid residues that are not essential for GFRα-X activity. Such GFRα-X proteins differ in amino acid sequence

from SEQ ID NO:2 yet retain at least one of the GFRα-X activities described herein. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:2 and is capable of binding a neurotrophic factor and modulating a response in a neurotrophic factor responsive cell. Preferably, the protein encoded by the nucleic acid molecule is at least about 70% homologous to SEQ ID NO:2, more preferably at least about 80-85% homologous to SEQ ID NO:2, even more preferably at least about 90% homologous to SEQ ID NO:2, and most preferably at least about 95-99% homologous to SEQ ID NO:2.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (e.g., when aligning a second sequence to the GFRα-X amino acid sequence of SEQ ID NO:2 having 177 amino acid residues, at least 80, preferably at least 100, more preferably at least 120, even more preferably at least 140, and even more preferably at least 150, 160 or 170 amino acid residues are aligned). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to GFRα-X nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to GFRα-X protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

An isolated nucleic acid molecule encoding a GFRα-X protein homologous to the protein of SEQ ID NO:2 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO:1 by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in GFRα-X is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a GFRα-X coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for a GFRα-X activity described herein to identify mutants that retain GFRα-X activity. Following mutagenesis of SEQ ID NO:1, the encoded protein can be expressed recombinantly (e.g., as described in Examples 4 and 5) and the activity of the protein can be determined using, for example, assays described herein.

In addition to the nucleic acid molecules encoding GFRα-X proteins described above, another aspect of the invention pertains to isolated nucleic acid molecules which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or

complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire GFRα-X coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding GFRα-X. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. Given the coding strand sequences encoding GFRα-X disclosed herein (e.g., SEQ ID NO:1), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of GFRα-X encoding mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of GFRα-X encoding mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of GFRα-X encoding mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-

methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a GFRα-X protein to thereby inhibit expression of the protein, e.g., by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementary to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of an antisense nucleic acid molecule of the invention includes direct injection at a tissue site. Alternatively, an antisense nucleic acid molecule can be modified to target selected cells and then administered systemically. For example, for systemic administration, an antisense molecule can be modified such that it specifically binds to a receptor or an antigen expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecule to a peptide or an antibody which binds to a cell surface receptor or antigen. The antisense nucleic acid molecule can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier et al. (1987) *Nucleic Acids.*

Res. 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al. (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analog (Inoue et al. (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (e.g., hammerhead ribozymes (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave GFRα-X encoding mRNA transcripts to thereby inhibit translation of GFRα-X encoding mRNA. A ribozyme having specificity for a GFRα-X-encoding nucleic acid can be designed based upon the nucleotide sequence of a *GFRα-X* cDNA disclosed herein (i.e., SEQ ID NO:1). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a GFRα-X-encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071 and Cech et al. U.S. Patent No. 5,116,742. Alternatively, GFRα-X encoding mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

Alternatively, *GFRα-X* gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the *GFRα-X* gene (e.g., the GFRα-X promoter and/or enhancers) to form triple helical structures that prevent transcription of the *GFRα-X* gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. et al. (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioassays* 14(12):807-15.

II. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding GFRα-X (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid",

which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art

that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., GFRα-X proteins, mutant forms of GFRα-X, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of GFRα-X in prokaryotic or eukaryotic cells. For example, GFRα-X can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. In one embodiment, the coding sequence of the GFRα-X is cloned into a pGEX

expression vector to create a vector encoding a fusion protein comprising, from the N-terminus to the C-terminus, GST-thrombin cleavage site-GFRα-X. The fusion protein can be purified by affinity chromatography using glutathione-agarose resin. Recombinant GFRα-X unfused to GST can be recovered by cleavage of the fusion protein with thrombin.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the GFRα-X expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, GFRα-X can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to GFRα-X encoding mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, GFRα-X protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized

techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding GFRα-X or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (i.e., express) GFRα-X protein. Accordingly, the invention further provides methods for producing GFRα-X protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding GFRα-X has been introduced) in a suitable medium until GFRα-X is produced. In another embodiment, the method further comprises isolating GFRα-X from the medium or the host cell.

The host cells of the invention can also be used to produce non-human transgenic animals. The non-human transgenic animals can be used in screening assays designed to identify agents or compounds, e.g., drugs, pharmaceuticals, etc., which are capable of ameliorating detrimental symptoms of selected disorders such as neurological disorders and morphological disorders. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which GFRα-X-coding

sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous GFRα-X sequences have been introduced into their genome or homologous recombinant animals in which endogenous GFRα-X sequences have been altered. Such animals are useful for studying the function and/or activity of GFRα-X and for identifying and/or evaluating modulators of GFRα-X activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous *GFRα-X* gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing GFRα-X-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The human homologue of the mouse *GFRα-X* nucleic acid molecule of SEQ ID NO:1 can be isolated based on hybridization to the mouse *GFRα-X* cDNA (described further in subsection I above) and used as a transgene, e.g., introduced as a transgene into the genome of a non-human animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the GFRα-X transgene to direct expression of GFRα-X protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both

by Leder et al., U.S. Patent No. 4,873,191 by Wagner et al. and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the GFRα-X transgene in its genome and/or expression of GFRα-X encoding mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding GFRα-X can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a *GFRα-X* gene into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the *GFRα-X* gene. The *GFRα-X* gene can be a human gene (e.g., from a human genomic clone isolated from a human genomic library screened with the cDNA of SEQ ID NO:1), but more preferably, is a non-human homologue of a human *GFRα-X* gene. For example, the mouse *GFRα-X* gene can be used to construct a homologous recombination vector suitable for altering an endogenous *GFRα-X* gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous *GFRα-X* gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous *GFRα-X* gene is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous GFRα-X protein). In the homologous recombination vector, the altered portion of the *GFRα-X* gene is flanked at its 5' and 3' ends by additional nucleic acid of the *GFRα-X* gene to allow for homologous recombination to occur between the exogenous *GFRα-X* gene carried by the vector and an endogenous *GFRα-X* gene in an embryonic stem cell. The additional flanking *GFRα-X* nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas, K.R. and

Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced *GFRα-X* gene has homologously recombined with the endogenous *GFRα-X* gene are selected (see e.g., Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec et al.; WO 91/01140 by Smithies et al.; WO 92/0968 by Zijlstra et al.; and WO 93/04169 by Berns et al.

In another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, e.g., Lakso et al. (1992) *PNAS* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. et al. (1997) *Nature* 385:810-813 and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit

the growth cycle and enter G_0 phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

III. Isolated GFRα-X Proteins and Anti-GFRα-X Antibodies

Another aspect of the invention pertains to isolated GFRα-X proteins, and biologically active portions thereof, as well as peptide fragments suitable for use as immunogens to raise anti-GFRα-X antibodies. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of GFRα-X protein in which the protein is separated from cellular components of the cells in which it is naturally or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of GFRα-X protein having less than about 30% (by dry weight) of non-GFRα-X protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-GFRα-X protein, still more preferably less than about 10% of non-GFRα-X protein, and most preferably less than about 5% non-GFRα-X protein. When the GFRα -X protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation. The language "substantially free of chemical precursors or other chemicals" includes preparations of GFRα-X protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of GFRα-X protein having less than about 30% (by dry weight) of chemical precursors or non-GFRα

-X chemicals, more preferably less than about 20% chemical precursors or non-GFRα-X chemicals, still more preferably less than about 10% chemical precursors or non-GFRα-X chemicals, and most preferably less than about 5% chemical precursors or non-GFRα-X chemicals. In preferred embodiments, isolated proteins or biologically active portions thereof lack contaminating proteins from the same animal from which the GFRα-X protein is derived. Typically, such proteins are produced by recombinant expression of, for example, a human GFRα-X protein in a non-human cell.

Preferably, an isolated GFRα-X protein or a portion thereof of the invention can bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell. In preferred embodiments, the protein or portion thereof comprises an amino acid sequence which is sufficiently homologous to an amino acid sequence of SEQ ID NO:2 such that the protein or portion thereof maintains the ability to bind a neurotrophic factor and modulate a response in a neurotrophic factor responsive cell. The portion of the protein is preferably a biologically active portion as described herein. In still another preferred embodiment, the GFRα-X protein has an amino acid sequence which is encoded by a nucleotide sequence that is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the nucleotide sequence of the DNA SEQ ID NO:1. The preferred GFRα-X proteins of the present invention also preferably possess at least one of the GFRα-X activities described herein.

In other embodiments, the GFRα-X protein is substantially homologous to the amino acid sequence of SEQ ID NO:2 and retains the functional activity of the protein of SEQ ID NO:2 yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail in subsection I above. Accordingly, in another embodiment, the GFRα-X protein is a protein which comprises an amino acid sequence which is at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to the entire amino acid sequence of SEQ ID NO:2 and which has at least one of the GFRα-X activities described herein. In other embodiment, the invention pertains to a protein which is substantially homologous to the entire amino acid sequence of SEQ ID NO:2.

Biologically active portions of the GFRα-X protein include peptides comprising amino acid sequences derived from the amino acid sequence of the GFRα-X protein, e.g., the amino acid sequence shown in SEQ ID NO:2 or the amino acid sequence of a protein homologous to the GFRα-X protein, which include less amino acids than the GFRα-X protein or the full length protein which is homologous to the GFRα-X protein, and exhibit at least one activity of the GFRα-X protein. Typically, biologically active portions (peptides, e.g., peptides which are, for example, 5, 10, 15, 20, 30, 35, 36, 37, 38, 39, 40, 50, 100 or more amino acids in length) comprise a domain or motif, e.g., an N-terminal hydrophobic domain, with at least one activity of the GFRα-X protein. Preferably, the domain is an N-terminal hydrophobic domain derived from a human and is at least about 55-60%, preferably at least about 65-70%, even more preferably at least about 75-80%, and most preferably at least about 85-90% or more homologous to SEQ ID NO:2.

GFRα-X proteins are preferably produced by recombinant DNA techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector (as described above), the expression vector is introduced into a host cell (as described above) and the GFRα-X protein is expressed in the host cell. The GFRα-X protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a GFR α-X protein, polypeptide, or peptide can be synthesized chemically using standard peptide synthesis techniques. Moreover, native GFRα-X protein can be isolated from cells (e.g., neural cells), for example using an anti-GFRα-X antibody (described further below).

The invention also provides GFRα-X chimeric or fusion proteins. As used herein, a GFRα-X "chimeric protein" or "fusion protein" comprises a GFRα-X polypeptide operatively linked to a non-GFRα-X polypeptide. An "GFRα-X polypeptide" refers to a polypeptide having an amino acid sequence corresponding to GFRα-X, or a fragment thereof, whereas a "non-GFRα-X polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not

substantially homologous to the GFRα-X protein, e.g., a protein which is different from the GFRα-X protein and which is derived from the same or a different organism. Within the fusion protein, the term "operatively linked" is intended to indicate that the GFRα-X polypeptide and the non-GFRα-X polypeptide are fused in-frame to each other. The non-GFRα-X polypeptide can be fused to the N-terminus or C-terminus of the GFRα-X polypeptide. For example, in one embodiment the fusion protein is a GST-GFRα-X fusion protein in which the GFRα-X sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant GFRα-X.

Preferably, a GFRα-X chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). A GFRα-X-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the GFRα-X protein.

The present invention also pertains to homologues of the GFRα-X proteins which function as either a GFRα-X agonist (mimetic) or a GFRα-X antagonist. In a preferred embodiment, the GFRα-X agonists and antagonists stimulate or inhibit, respectively, a subset of the biological activities of the naturally occurring form of the GFRα-X protein. Thus, specific biological effects can be elicited by treatment with a

homologue of limited function. In one embodiment, treatment of a subject with a homologue having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the GFRα-X protein.

Homologues of the GFRα-X protein can be generated by mutagenesis, e.g., discrete point mutation or truncation of the GFRα-X protein. As used herein, the term "homologue" refers to a variant form of the GFRα-X protein which acts as an agonist or antagonist of the activity of the GFRα-X protein. An agonist of the GFRα-X protein can retain substantially the same, or a subset, of the biological activities of the GFRα-X protein. An antagonist of the GFRα-X protein can inhibit one or more of the activities of the naturally occurring form of the GFRα-X protein, by, for example, competitively binding to a downstream or upstream member of the GFRα-X cascade which includes the GFRα-X protein. Thus, the mammalian GFRα-X protein and homologues thereof of the present invention can be either positive or negative regulators of neurotrophic factor responses in cells responsive to a neurotrophic factor.

In an alternative embodiment, homologues of the GFRα-X protein can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants, of the GFRα-X protein for GFRα-X protein agonist or antagonist activity. In one embodiment, a variegated library of GFRα-X variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of GFRα-X variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential GFRα-X sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of GFRα-X sequences therein. There are a variety of methods which can be used to produce libraries of potential GFRα-X homologues from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one

mixture, of all of the sequences encoding the desired set of potential GFRα-X sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of the GFRα-X protein coding can be used to generate a variegated population of GFRα-X fragments for screening and subsequent selection of homologues of a GFRα-X protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a GFRα-X coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the GFRα-X protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of GFR α-X homologues. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recrusive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify GFRα-X homologues (Arkin and Yourvan (1992) *PNAS 89*:7811-7815; Delgrave et al. (1993) *Protein Engineering* 6(3):327-331).

In one embodiment, cell based assays can be exploited to analyze a variegated GFRα-X library. For example, a library of expression vectors can be transfected into a cell line ordinarily responsive to a particular neurotrophic factor. The transfected cells are then contacted with the neurotrophic factor and the effect of the GFRα-X mutant on signaling by the neurotrophic factor can be detected, e.g., by measuring ^{3}[H]thymidine incorporation. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of neurotrophic factor induction, and the individual clones further characterized.

An isolated GFRα-X protein, or a portion or fragment thereof (particularly fragments comprising residues displaying high antigenicity scores, Fig. 3), can be used as an immunogen to generate antibodies that bind GFRα-X using standard techniques for polyclonal and monoclonal antibody preparation. The GFRα-X protein of SEQ ID NO:2 can be used or, alternatively, the invention provides antigenic peptide fragments of GFRα-X for use as immunogens. The antigenic peptide of GFRα-X comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of GFRα-X such that an antibody raised against the peptide forms a specific immune complex with GFRα-X. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of GFRα-X that are located on the surface of the protein, e.g., hydrophilic regions.

A GFRα-X immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed GFRα-X protein or a chemically synthesized GFRα-X peptide. Prefered fragments of GFRα-X for use as an immunogen are fragments comprising high antigenicity scores shown in Figue 2 and conserved regions of high homology shown in Figure 3.. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable

subject with an immunogenic GFRα-X preparation induces a polyclonal anti-GFRα-X antibody response.

Accordingly, another aspect of the invention pertains to anti-GFRα-X antibodies. Preferably the antibodies of the present invention will bind GFRα-X but will not bind GFRα-1, GFRα-2, or GFRα-3. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as GFRα-X. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind GFRα-X. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of GFRα-X. A monoclonal antibody composition thus typically displays a single binding affinity for a particular GFRα-X protein with which it immunoreacts.

Polyclonal anti-GFRα-X antibodies can be prepared as described above by immunizing a suitable subject with a GFRα-X immunogen. The anti-GFRα-X antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized GFRα-X. If desired, the antibody molecules directed against GFRα-X can be isolated from the mammal (e.g., from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-GFRα-X antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown et al. (1981) *J. Immunol.* 127:539-46; Brown et al. (1980) *J. Biol. Chem* .255:4980-83; Yeh et al. (1976) *PNAS* 76:2927-31; and Yeh et al. (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72),

the EBV-hybridoma technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter et al. (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a GFRα-X immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds GFRα-X.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-GFRα-X monoclonal antibody (see, e.g., G. Galfre et al. (1977) *Nature* 266:55052; Gefter et al. *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody

of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind GFRα-X, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-GFRα-X antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with GFRα-X to thereby isolate immunoglobulin library members that bind GFRα-X. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. PCT International Publication No. WO 92/18619; Dower et al. PCT International Publication No. WO 91/17271; Winter et al. PCT International Publication WO 92/20791; Markland et al. PCT International Publication No. WO 92/15679; Breitling et al. PCT International Publication WO 93/01288; McCafferty et al. PCT International Publication No. WO 92/01047; Garrard et al. PCT International Publication No. WO 92/09690; Ladner et al. PCT International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J. Mol. Biol.* 226:889-896; Clarkson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas et al. (1991) *PNAS* 88:7978-7982; and McCafferty et al. *Nature* (1990) 348:552-554.

Additionally, recombinant anti-GFRα-X antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson et al. International Application No. PCT/US86/02269; Akira, et al. European

Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al. European Patent Application 173,494; Neuberger et al. PCT International Publication No. WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al. European Patent Application 125,023; Better et al. (1988) *Science* 240:1041-1043; Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *PNAS* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi et al. (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060.

An anti-GFRα-X antibody (e.g., monoclonal antibody) can be used to isolate GFRα-X by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-GFRα-X antibody can facilitate the purification of natural GFRα-X from cells and of recombinantly produced GFRα-X expressed in host cells. Moreover, an anti-GFRα-X antibody can be used to detect GFRα-X protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the GFRα-X protein. Anti-GFRα-X antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent

materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

IV. Pharmaceutical Compositions

The *GFRα-X* nucleic acid molecules, GFRα-X proteins, fragments thereof, GFR α-X modulators, and anti-GFRα-X antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. Such compositions typically comprise the nucleic acid molecule, protein, modulator, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a GFRα-X protein, fragment, or anti-GFRα-X antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

V. Uses and Methods f the Invention

The nucleic acid molecules, proteins, protein homologues, modulators, and antibodies described herein can be used in one or more of the following methods: a) drug screening assays; b) diagnostic assays; c) methods of treatment; d) pharmacogenomics; and e) monitoring of effects during clinical trials. A GFRα-X protein of the invention has one or more of the activities described herein and can thus be used to, for example, bind a neurotrophic factor and modulate response in a neurotrophic factor responsive cell. The isolated nucleic acid molecules of the invention can be used to express GFRα-X protein (e.g., via a recombinant expression vector in a host cell in gene therapy applications), to detect GFRα-X encoding mRNA (e.g., in a biological sample) or a genetic mutation in a *GFRα-X* gene, and to modulate GFRα-X activity, as described further below. In addition, the GFRα-X proteins can be used to screen drugs or compounds which modulate GFRα-X protein activity as well as to treat disorders characterized by insufficient production of GFRα-X protein or production of GFRα-X protein forms which have decreased activity compared to wild type GFRα-X. Moreover, the anti-GFRα-X antibodies of the invention can be used to detect and isolate GFRα-X protein and modulate GFRα-X protein activity.

a. Drug Screening Assays

The invention provides methods for identifying compounds or agents that can be used to treat disorders characterized by (or associated with) aberrant or abnormal *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. These methods are also referred to herein as drug screening assays and typically include the step of screening a candidate/test compound or agent for the ability to interact with (e.g., bind to) a GFRα-X protein, to modulate the interaction of a GFRα-X protein and a target molecule, and/or to modulate *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. Candidate/test compounds or agents which have one or more of these abilities can be used as drugs to treat disorders characterized by aberrant or abnormal *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. Candidate/test compounds include, for

example, 1) peptides such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, e.g., Lam, K.S. et al. (1991) *Nature* 354:82-84; Houghten, R. et al. (1991) *Nature* 354:84-86) and combinatorial chemistry-derived molecular libraries made of D- and/or L- configuration amino acids; 2) phosphopeptides (e.g., members of random and partially degenerate, directed phosphopeptide libraries, see, e.g., Songyang, Z. et al. (1993) *Cell* 72:767-778); 3) antibodies (e.g., polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, $F(ab')_2$, Fab expression library fragments, and epitope-binding fragments of antibodies); and 4) small organic and inorganic molecules (e.g., molecules obtained from combinatorial and natural product libraries).

In one embodiment, the invention provides assays for screening candidate/test compounds which interact with (e.g., bind to) GFRα-X protein. Typically, the assays are cell-based assays which include the steps of combining a GFRα-X protein, a biologically active portion thereof, or a cell expressing GFRα-X protein or fragment thereof, and a candidate/test compound, e.g., under conditions which allow for interaction of (e.g., binding of) the candidate/test compound to the GFRα-X protein or portion thereof to form a complex, and detecting the formation of a complex, in which the ability of the candidate compound to interact with (e.g., bind to) the GFRα-X protein or portion thereof is indicated by the presence of the candidate compound in the complex. Formation of complexes between the GFRα-X protein and the candidate compound can be quantitated, for example, using standard immunoassays.

In another embodiment, the invention provides screening assays to identify candidate/test compounds which modulate (e.g., stimulate or inhibit) the interaction (and most likely GFRα-X activity as well) between a GFRα-X protein and a molecule (target molecule) with which the GFRα-X protein normally interacts. Examples of such target molecules includes proteins in the same signaling path as the GFRα-X protein, e.g., proteins which may function upstream (including both stimulators and inhibitors of activity) e.g., GDNF and/or NTN or downstream of the GFRα-X protein in the neurotrophic factor signaling pathway, e.g., the tyrosine kinase RET receptor. Typically, the assays are cell-based assays which include the steps of combining a GFR

α-X protein, a biologically active portion thereof, or a cell expressing GFRα-X protein or fragment thereof, a GFRα-X target molecule (e.g., a GFRα-X ligand) and a candidate/test compound, e.g., under conditions wherein but for the presence of the candidate compound e.g., GDNF or NTN, the GFRα-X protein or biologically active portion thereof interacts with (e.g., binds to) the target molecule, and detecting the formation of a complex which includes the GFRα-X protein and the target molecule or detecting the interaction/reaction of the GFRα-X protein and the target molecule. Detection of complex formation can include direct quantitation of the complex by, for example, measuring inductive effects of the GFRα-X protein. A statistically significant change, such as a decrease, in the interaction of the GFRα-X and target molecule (e.g., in the formation of a complex between the GFRα-X and the target molecule) in the presence of a candidate compound (relative to what is detected in the absence of the candidate compound) is indicative of a modulation (e.g., stimulation or inhibition) of the interaction between the GFRα-X protein and the target molecule. Modulation of the formation of complexes between the GFRα-X protein and the target molecule can be quantitated using, for example, an immunoassay.

To perform the above drug screening assays, it may be desirable to immobilize either GFRα-X or its target molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Interaction (e.g., binding of) of GFRα-X to a target molecule, in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/ GFRα-X fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the cell lysates (e.g. ^{35}S-labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads are washed to remove any unbound label, and the matrix

immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of GFRα-X-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices can also be used in the drug screening assays of the invention. For example, either GFRα-X or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated GFRα-X molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with GFRα-X but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and GFRα-X trapped in the wells by antibody conjugation. As described above, preparations of a GFRα-X-binding protein and a candidate compound are incubated in the GFRα-X-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the GFRα-X target molecule, or which are reactive with GFRα-X protein and compete with the target molecule; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the target molecule.

In yet another embodiment, the invention provides a method for identifying a compound (e.g., a screening assay) capable of use in the treatment of a disorder characterized by (or associated with) aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity. This method typically includes the step of assaying the ability of the compound or agent to modulate the expression of the *GFRα-X* nucleic acid or the activity of the GFRα-X protein thereby identifying a compound for treating a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity. Disorders characterized by aberrant or abnormal

GFRα-X nucleic acid expression or GFRα-X protein activity are described herein. Methods for assaying the ability of the compound or agent to modulate the expression of the *GFRα-X* nucleic acid or activity of the GFRα-X protein are typically cell-based assays. For example, cells which are sensitive to ligands, e.g., GDNF, which transduce signals via a pathway involving GFRα-X can be induced to overexpress a GFRα-X protein in the presence and absence of a candidate compound. Candidate compounds which produce a statistically significant change in GFRα-X-dependent responses (either stimulation or inhibition) can be identified. In one embodiment, expression of the *GFR α-X* nucleic acid or activity of a GFRα-X protein is modulated in cells and the effects of candidate compounds on the readout of interest (such as rate of cell proliferation or differentiation) are measured. For example, the expression of genes which are up- or down-regulated in response to a GFRα-X-dependent signal cascade can be assayed. In preferred embodiments, the regulatory regions of such genes, e.g., the 5' flanking promoter and enhancer regions, are operably linked to a detectable marker (such as luciferase) which encodes a gene product that can be readily detected. Phosphorylation of GFRα-X or GFRα-X target molecules can also be measured, for example, by immunoblotting.

Alternatively, modulators of GFRα-X expression (e.g., compounds which can be used to treat a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity) can be identified in a method wherein a cell is contacted with a candidate compound and the expression of GFRα-X encoding mRNA or protein in the cell is determined. The level of expression of GFRα-X encoding mRNA or protein in the presence of the candidate compound is compared to the level of expression of GFRα-X encoding mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of *GFRα-X* nucleic acid expression based on this comparison and be used to treat a disorder characterized by aberrant *GFRα-X* nucleic acid expression. For example, when expression of GFRα-X encoding mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate

compound is identified as a stimulator of GFRα-X encoding mRNA or protein expression. Alternatively, when expression of GFRα-X encoding mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of GFRα-X encoding mRNA or protein expression. The level of GFRα-X encoding mRNA or protein expression in the cells can be determined by methods described herein for detecting GFRα-X encoding mRNA or protein.

In yet another aspect of the invention, the GFRα-X proteins, particularly fragments of GFRα-X, can be used as "bait proteins" in a two-hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J. Biol. Chem.* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; Iwabuchi et al. (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with GFRα-X ("GFRα-X-binding proteins" or "GFR α-X-bp") and modulate GFRα-X protein activity. Such GFRα-X-binding proteins are also likely to be involved in the propagation of signals by the GFRα-X proteins as, for example, upstream or downstream elements of the GFRα-X pathway.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for GFRα-X is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming a GFRα-X-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcription regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with GFRα-X.

Modulators of GFRα-X protein activity and/or *GFRα-X* nucleic acid expression identified according to these drug screening assays can be to treat, for example, neurological diseases or disorders described herein. These methods of treatment include the steps of administering the modulators of GFRα-X protein activity and/or nucleic acid expression, e.g., in a pharmaceutical composition as described in subsection IV above, to a subject in need of such treatment, e.g., a subject with a neurological disease.

b. Diagnostic Assays

The invention further provides a method for detecting the presence of GFRα-X in a biological sample. The method involves contacting the biological sample with a compound or an agent capable of detecting GFRα-X protein or mRNA such that the presence of GFRα-X is detected in the biological sample. A preferred agent for detecting GFRα-X encoding mRNA is a labeled or labelable nucleic acid probe capable of hybridizing to GFRα-X encoding mRNA. The nucleic acid probe can be, for example, the *GFRα-X* cDNA of SEQ ID NO:1, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to GFRα-X encoding mRNA. A preferred agent for detecting GFRα-X protein is a labeled or labelable antibody capable of binding to GFRα-X protein. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) can be used. The term "labeled or labelable", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect GFRα-X encoding mRNA or protein in a

biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of GFRα-X encoding mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of GFRα-X protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. Alternatively, GFRα-X protein can be detected *in vivo* in a subject by introducing into the subject a labeled anti-GFRα-X antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques. In one preferred embodiment of the detection method, the biological sample is a neural cell sample. The neural cell sample can comprise neural tissue or a suspension of neural cells. A tissue section, for example, a freeze-dried or fresh frozen section of neural tissue removed from a patient, can be used as the neural cell sample. Alternatively, the biological sample can comprise a biological fluid (e.g., cerebrospinal fluid) obtained from a subject having a neurological disorder. In another preferred embodiment of the detection method, the biological sample is a neural cell sample (e.g., a sample which includes motorneuron cells). The invention also encompasses kits for detecting the presence of GFRα-X in a biological sample. For example, the kit can comprise a labeled or labelable compound or agent capable of detecting GFRα-X protein or mRNA in a biological sample; means for determining the amount of GFRα-X in the sample; and means for comparing the amount of GFRα-X in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect GFRα-X encoding mRNA or protein.

The methods of the invention can also be used to detect genetic mutations in a *GFRα-X* gene, or the allelic form of *GFRα-X* found in a subject, thereby determining if a subject with the mutated gene is at risk for a disorder characterized by aberrant or abnormal *GFRα-X* nucleic acid expression or GFRα-X protein activity as defined herein. In preferred embodiments, the methods include detecting, in a sample from the subject, the presence or absence of a genetic mutation characterized by at least one of an alteration affecting the integrity of a gene encoding a GFRα-X protein, or the misexpression of the *GFRα-X* gene. For example, such genetic mutations can be

detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a *GFRα-X* gene; 2) an addition of one or more nucleotides to a *GFRα-X* gene; 3) a substitution of one or more nucleotides of a *GFRα-X* gene, 4) a chromosomal rearrangement of a *GFRα-X* gene; 5) an alteration in the level of a messenger RNA transcript of a *GFRα-X* gene, 6) aberrant modification of a *GFRα-X* gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a *GFRα-X* gene, 8) a non-wild type level of a GFRα-X-protein, 9) allelic loss of a *GFRα-X* gene, and 10) inappropriate post-translational modification of a GFRα-X-protein. As described herein, there are a large number of assay techniques known in the art which can be used for detecting mutations in a *GFRα-X* gene.

In certain embodiments, detection of the mutation involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) *Science* 241:1077-1080; and Nakazawa et al. (1994) *PNAS* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the GFRα-X-gene (see Abravaya et al. (1995) *Nucleic Acids Res* .23:675-682). This method can include the steps of collecting a sample from a patient, isolating nucleic acid (e.g., genomic, mRNA or both) from the sample, contacting the nucleic acid with one or more primers which specifically hybridize to a *GFRα-X* gene under conditions such that hybridization and amplification of the *GFRα-X*-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In an alternative embodiment, mutations in a *GFRα-X* gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence

specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the *GFRα-X* gene and detect mutations by comparing the sequence of the sample *GFRα-X* with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxim and Gilbert ((1977) *PNAS* 74:560) or Sanger ((1977) *PNAS* 74:5463). A variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO 94/16101; Cohen et al. (1996) *Adv. Chromatogr.* 36:127-162; and Griffin et al. (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the *GFRα-X* gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al. (1985) *Science* 230:1242); Cotton et al. (1988) *PNAS* 85:4397; Saleeba et al. (1992) *Meth. Enzymol.* 217:286-295), electrophoretic mobility of mutant and wild type nucleic acid is compared (Orita et al. (1989) *PNAS* 86:2766; Cotton (1993) *Mutat Res* 285:125-144; and Hayashi (1992) *Genet Anal Tech Appl* 9:73-79), and movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (Myers et al (1985) *Nature* 313:495). Examples of other techniques for detecting point mutations include, selective oligonucleotide hybridization, selective amplification, and selective primer extension.

c. Methods of Treatment

Another aspect of the invention pertains to methods for treating a subject, e.g., a human, having a disease or disorder characterized by (or associated with) aberrant or abnormal *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. These methods include the step of administering a GFRα-X modulator to the subject such that

treatment occurs. The language "aberrant or abnormal *GFRα-X* expression" refers to expression of a non-wild-type GFRα-X protein or a non-wild-type level of expression of a GFRα-X protein. Aberrant or abnormal GFRα-X activity refers to a non-wild-type GFRα-X activity or a non-wild-type level of GFRα-X activity. As the GFRα-X protein is involved in a pathway involving neurological and developmental functions, aberrant or abnormal GFRα-X protein activity or nucleic acid expression interferes with normal neurological functions and/or developmental functions. Non-limiting examples of neurological disorders or diseases characterized by or associated with abnormal or aberrant GFRα-X protein activity or nucleic acid expression in neural cells include sensory, disorders, e.g., Dejerine-Roussy Syndrome, motor disorders, e.g., Parkinson's disease, ALS, and cognitive disorders, e.g., Alzheimer's disease. Examples of disorders or diseases characterized by or associated with abnormal or aberrant GFRα-X protein activity or nucleic acid expression in cells associated with developmental function include disorders of the enteric nervous system, e.g., Hirschsprung's disease and eating disorders.

The terms "treating" or "treatment", as used herein, refer to reduction or alleviation of at least one adverse effect or symptom of a disorder or disease, e.g., a disorder or disease characterized by or associated with abnormal or aberrant GFRα-X protein activity or *GFRα-X* nucleic acid expression.

As used herein, a GFRα-X modulator is a molecule which can modulate *GFRα-X* nucleic acid expression and/or GFRα-X protein activity. For example, a GFRα-X modulator can modulate, e.g., upregulate (activate) or downregulate (suppress), *GFRα-X* nucleic acid expression. In another example, a GFRα-X modulator can modulate (e.g., stimulate or inhibit) GFRα-X protein activity. If it is desirable to treat a disorder or disease characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFR α-X* nucleic acid expression and/or GFRα-X protein activity by inhibiting *GFRα-X* nucleic acid expression, a GFRα-X modulator can be an antisense molecule, e.g., a ribozyme, as described herein. Examples of antisense molecules which can be used to inhibit *GFRα-X* nucleic acid expression include antisense molecules which are

complementary to a portion of the 5' untranslated region of the GFRα-X encoding sequence which also includes the start codon and antisense molecules which are complementary to a portion of the 3' untranslated region.

A GFRα-X modulator which inhibits *GFRα-X* nucleic acid expression can also be a small molecule or other drug, e.g., a small molecule or drug identified using the screening assays described herein, which inhibits *GFRα-X* nucleic acid expression. If it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFRα-X* nucleic acid expression and/or GFRα-X protein activity by stimulating *GFRα-X* nucleic acid expression, a GFRα-X modulator can be, for example, a nucleic acid molecule encoding GFRα-X (e.g., a nucleic acid molecule comprising a nucleotide sequence homologous to the nucleotide sequence of SEQ ID NO:1) or a small molecule or other drug, e.g., a small molecule (peptide) or drug identified using the screening assays described herein, which stimulates *GFRα-X* nucleic acid expression.

Alternatively, if it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFRα-X* nucleic acid expression and/or GFRα-X protein activity by inhibiting GFRα-X protein activity, a GFRα-X modulator can be an anti-GFRα-X antibody or a small molecule or other drug, e.g., a small molecule or drug identified using the screening assays described herein, which inhibits GFRα-X protein activity. If it is desirable to treat a disease or disorder characterized by (or associated with) aberrant or abnormal (non-wild-type) *GFRα-X* nucleic acid expression and/or GFRα-X protein activity by stimulating GFRα-X protein activity, a GFRα-X modulator can be an active GFRα-X protein or portion thereof (e.g., a GFRα-X protein or portion thereof having an amino acid sequence which is homologous to the amino acid sequence of SEQ ID NO:2 or a portion thereof) or a small molecule or other drug, e.g., a small molecule or drug identified using the screening assays described herein, which stimulates GFRα-X protein activity.

In addition, a subject having a neurological disorder can be treated according to the present invention by administering to the subject a GFRα-X protein, preferably a portion thereof, or a nucleic acid encoding a GFRα-X protein or portion thereof such that treatment occurs. Similarly, a subject having a developmental disorder can be treated according to the present invention by administering to the subject a GFRα-X protein or portion thereof or a nucleic acid encoding a GFRα-X protein or portion thereof such that treatment occurs.

Other aspects of the invention pertain to methods for modulating a cell associated activity. These methods include contacting the cell with an agent (or a composition which includes an effective amount of an agent) which modulates GFRα-X protein activity or *GFRα-X* nucleic acid expression such that a cell associated activity is altered relative to a cell associated activity of the cell in the absence of the agent. As used herein, "a cell associated activity" refers to a normal or abnormal activity or function of a cell. Examples of cell associated activities include proliferation, migration, differentiation, production or secretion of molecules, such as proteins, and cell survival. In a preferred embodiment, the cell is neural cell of the CNS, e.g., motorneuron of the spinal cord. The term "altered" as used herein refers to a change, e.g., an increase or decrease, of a cell associated activity. In one embodiment, the agent stimulates GFRα-X protein activity or *GFRα-X* nucleic acid expression. Examples of such stimulatory agents include an active GFRα-X protein, a nucleic acid molecule encoding GFRα-X that has been introduced into the cell, and a modulatory agent which stimulates GFR-Xα protein activity or *GFRα-X* nucleic acid expression and which is identified using the drug screening assays described herein. In another embodiment, the agent inhibits GFR α-X protein activity or *GFRα-X* nucleic acid expression. Examples of such inhibitory agents include an antisense *GFRα-X* nucleic acid molecule, an anti-GFR-Xα antibody, and a modulatory agent which inhibits GFRα-X protein activity or *GFRα-X* nucleic acid expression and which is identified using the drug screening assays described herein. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject). In a preferred embodiment, the modulatory methods are performed *in vivo*, i.e., the cell is

present within a subject, e.g., a mammal, e.g., a human, and the subject has a disorder or disease characterized by or associated with abnormal or aberrant GFRα-X protein activity or *GFRα-X* nucleic acid expression.

A nucleic acid molecule, a protein, a GFRα-X modulator, a compound etc. used in the methods of treatment can be incorporated into an appropriate pharmaceutical composition described herein and administered to the subject through a route which allows the molecule, protein, modulator, or compound etc. to perform its intended function. Examples of routes of administration are also described herein under subsection IV.

d. Pharmacogenomics

Test/candidate compounds, or modulators which have a stimulatory or inhibitory effect on GFRα-X activity (e.g., *GFRα-X* gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (e.g., neural disorders, e.g., central and peripheral nervous system disorders) associated with aberrant GFRα-X activity. In conjunction with such treatment, the pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permit the selection of effective compounds (e.g., drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly, the activity of GFRα-X polypeptide, expression of *GFRα-X* nucleic acid, or mutation content of *GFRα-X* genes in an individual can be determined to thereby select appropriate compound(s) for therapeutic or prophylactic treatment of the individual.

Pharmacogenomics deal with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Eichelbaum, M. (1996) *Clin. Exp. Pharmacol. Physiol.* 23(10-11):983-985 and Linder, M.W. (1997) *Clin. Chem.* 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus, the activity of GFRα-X polypeptide, expression of *GFRα-X* nucleic acid, or mutation content of *GFRα-X* genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of a subject. In addition, pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of a subject's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with an GFRα-X modulator, such as a modulator identified by one of the exemplary screening assays described herein.

e. **Monitoring of Effects During Clinical Trials**

Monitoring the influence of compounds (e.g., drugs) on the expression or activity of GFRα-X (e.g., the ability to modulate the effects of neurotrophic factors on neurotrophic factor responsive cells) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay, as described herein, to increase *GFRα-X* gene expression, polypeptide levels, or up-regulate GFRα-X activity, can be monitored in clinical trials of subjects exhibiting decreased *GFRα-X* gene expression, polypeptide levels, or down-regulated GFRα-X activity. Alternatively, the effectiveness of an agent, determined by a screening assay, to decrease *GFRα-X* gene expression, polypeptide levels, or down-regulate GFRα-X activity, can be monitored in clinical trials of subjects exhibiting increased *GFRα-X* gene expression, polypeptide levels, or up-regulated GFRα-X activity. In such clinical trials, the expression or activity of GFRα-X and, preferably, other genes which have been implicated in, for example, a neural disorder, e.g., a central nervous system disorder, can be used as a "read out" or markers of the neurotrophic factor responsiveness of a particular cell.

For example, and not by way of limitation, genes, including GFRα-X, which are modulated in cells by treatment with a compound (e.g., drug or small molecule) which modulates GFRα-X activity (e.g., identified in a screening assay as described herein) can be identified. Thus, to study the effect of compounds on neural disorders, e.g.,

central and peripheral nervous system disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of GFRα-X and other genes implicated in the disorder. The levels of gene expression (i.e., a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of polypeptide produced, by one of the methods described herein, or by measuring the levels of activity of GFRα-X or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the compound. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the compound.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with a compound (e.g., an agonist, antagonist, peptidomimetic, polypeptide, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the compound; (ii) detecting the level of expression of an GFRα-X polypeptide, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the GFRα-X polypeptide, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the GFRα-X polypeptide, mRNA, or genomic DNA in the pre-administration sample with the GFRα-X polypeptide, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the compound to the subject accordingly. For example, increased administration of the compound may be desirable to increase the expression or activity of GFRα-X to higher levels than detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of GFRα-X to lower levels than detected, i.e., to decrease the effectiveness of the compound.

VI. Uses of Partial GFRα-X Sequences

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (a) map their respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (b) identify an individual from a minute biological sample (tissue typing); and (c) aid in forensic identification of a biological sample. These applications are described in the subsections below.

a. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. This process is called chromosome mapping. Accordingly, portions or fragments of the GFRα-X sequence, described herein, can be used to map the location of the *GFRα-X* gene, respectively, on a chromosome. The mapping of the *GFRα-X* sequence to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, the *GFRα-X* gene can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the GFRα-X sequences. Computer analysis of the

GFRα-X sequence can be used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the GFRα-X sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (e.g., human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains

the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio P. et al. (1983) *Science* 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the GFRα-X sequence to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a GFRα-X sequence to its chromosome include *in situ* hybridization (described in Fan, Y. et al. (1990) *PNAS*, 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical like colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma et al., Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York 1988).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to

noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. et al. (1987) *Nature* 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the *GFRα-X* gene can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

b. Tissue Typing

The GFRα-X sequence of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the GFRα-X sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The GFRα-X sequence of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1 can comfortably provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If the predicted coding sequence, such as the one in SEQ ID NO:2 is used, a more appropriate number of primers for positive individual identification would be 500-2,000.

If a panel of reagents from GFRα-X sequence described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

c. Use of Partial GFRα-X Sequence in Forensic Biology

DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, e.g., PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (i.e., another DNA sequence that is unique to a particular individual). As discussed above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the GFRα-X sequence or portions thereof, e.g., fragments derived from the noncoding regions of SEQ ID NO:1 having a length of at least 20 bases, preferably at least 30 bases.

The GFRα-X sequences described herein can further be used to provide polynucleotide reagents, e.g., labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, e.g., brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such GFRα-X probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, e.g., GFRα-X primers or probes can be used to screen tissue culture for contamination (i.e., screen for the presence of a mixture of different types of cells in a culture).

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patent applications, patents, and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

EXAMPLE 1: IDENTIFICATION OF MOUSE *GFRα-X* CDNA

In this example, the GFRα-X mouse cDNA was identified in a positional cloning process in which the mouse mahogany locus was being sequenced.

EXAMPLE 2: IDENTIFICATION OF HUMAN *GFRα-X* cDNA

To obtain the human *GFRα-X* nucleic acid molecule, a cDNA library from a human brain cell library (available from Stratagene, LaJolla, CA, or Clontech, Palo Alto, CA) is screened under low stringency conditions (e.g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) using a probe comprising the nucleotide sequence of SEQ ID NO:1 or a portion thereof. Clones obtained from this screen are sequenced and compared to the mouse sequence shown in SEQ ID NO:1 to determine if they are the human GFRα-X molecule. If the clones are found to be partial clones, the cDNA library is rescreened with the partial human clone to obtain the full length human clone.

EXAMPLE 3:TISSUE EXPRESSION OF THE MOUSE GRFα-X GENE

Northern Analysis Using RNA from Human Tissue

Mouse multiple tissue northern blots (Stratagene, Palo Alto, CA) containing 2 μg of poly A+ RNA per lane were probed with probes based on SEQ ID No:1. The filters were prehybridized in 5 ml of Church buffer at 65°C for 1 hour, after which 100 ng of ^{32}P labeled probe was added. The probe was generated using the Stratagene Prime-It kit, Catalog Number 300392 (Clontech, Palo Alto, CA). Hybridization was allowed to

proceed at 65°C for approximately 20 hours. The filters were washed in 0.1% SDS, 0.2 X SSC solution at 65°C and then exposed to the phosphoimager for 4 hours. The rat tissues tested included: heart, brain, spleen, lung, liver, stomach, kidney, and testis.

There was strong hybridization to the brain RNA represented in this Northern blot indicating that the *GFRα-X* gene transcript is expressed in brain. In situ hybridization showed expression in the Lateral septal neurons, Septohypothalamic neurons, paraventricular thalamic neurons (anterior), superchiasmatic neurons, anterior cortical amygdaloid neurons, piriform cortex, paracentral thalamic neurons, lateral habenular neurons, paraventricular hypothalamic neurons (PVN), amygdaloid nucleus area, arcuate neurons, and ventromedial hypothalamic neurons (VMH).

EXAMPLE 4: EXPRESSION OF RECOMBINANT GFRα-X PROTEIN IN COS CELLS

To express the *GFRα-X* gene in COS cells, the pcDNA/Amp vector by Invitrogen Corporation (San Diego, CA) is used. This vector contains an SV40 origin of replication, an ampicillin resistance gene, an *E. coli* replication origin, a CMV promoter followed by a polylinker region, and an SV40 intron and polyadenylation site. A DNA fragment encoding the entire GFRα-X protein and a HA tag (Wilson et al. (1984) *Cell* 37:767) fused in-frame to its 3' end of the fragment is cloned into the polylinker region of the vector, thereby placing the expression of the recombinant protein under the control of the CMV promoter.

To construct the plasmid, the GFRα-X DNA sequence is amplified by PCR using two primers. The 5' primer contains the restriction site of interest followed by approximately twenty nucleotides of the GFRα-X coding sequence starting from the initiation codon; the 3' end sequence contains complementary sequences to the other restriction site of interest, a translation stop codon, the HA tag and the last 20 nucleotides of the GFRα-X coding sequence. The PCR amplified fragment and the pCDNA/Amp vector are digested with the appropriate restriction enzymes and the vector is dephosphorylated using the CIAP enzyme (New England Biolabs, Beverly, MA). Preferably the two restriction sites chosen are different so that the *GFRα-X* gene

is inserted in the correct orientation. The ligation mixture is transformed into *E. coli* cells (strains HB101, DH5a, SURE, available from Stratagene Cloning Systems, La Jolla, CA, can be used), the transformed culture is plated on ampicillin media plates, and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment.

COS cells are subsequently transfected with the GFRα-X-pcDNA/Amp plasmid DNA using the calcium phosphate or calcium chloride co-precipitation methods, DEAE-dextran-mediated transfection, lipofection, or electroporation. Other suitable methods for transfecting host cells can be found in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The expression of the GFRα-X protein is detected by radiolabelling (^{35}S-methionine or ^{35}S-cysteine available from NEN, Boston, MA, can be used) and immunoprecipitation (Harlow, E. and Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988) using an HA specific monoclonal antibody. Briefly, the cells are labeled for 8 hours with ^{35}S-methionine (or ^{35}S-cysteine). The culture media are then collected and the cells are lysed using detergents (RIPA buffer, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM Tris, pH 7.5). Both the cell lysate and the culture media are precipitated with an HA specific monoclonal antibody. Precipitated proteins are then analyzed by SDS-PAGE.

Alternatively, DNA containing the GFRα-X coding sequence is cloned directly into the polylinker of the pCDNA/Amp vector using the appropriate restriction sites. The resulting plasmid is transfected into COS cells in the manner described above, and the expression of the GFRα-X protein is detected by radiolabelling and immunoprecipitation using a GFRα-X specific monoclonal antibody

EXAMPLE 5: CHARACTERIZATION OF GFRα-X PROTEIN

In this example, the amino acid sequence of the GFRα-X protein was compared to amino acid sequences of known proteins and various motifs were identified.

The GFRα-X protein, the amino acid sequence of which is shown in Figure 1 (SEQ ID NO:2), is a novel protein which includes 340 amino acid residues. A comparison of GFRα-X with other members of the GFR family of proteins is provided in Figure 3.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. An isolated nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule which encodes a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a nucleic acid molecule which encodes a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a nucleic acid molecule which encodes a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:2 under stringent conditions.

2. The nucleic acid molecule of claim 1 further comprising vector nucleic acid sequences.

3. The nucleic acid molecule of claim 1 further comprising nucleic acid sequences encoding a heterologous protein.

4. A host cell which contains the nucleic acid molecule of claim 1.

5. The host cell of claim 4 which is a mammalian host cell.

6. A non-human mammalian host cell containing the nucleic acid molecule of claim 1.

7. The isolated nucleic acid molecule of claim 1, which is selected from the group consisting of the coding region of SEQ ID NO:1 and the extracellular domain encoded by SEQ ID NO:1.

8. An isolated protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions.

9. The protein of claim 8 further comprising heterologous amino acid sequences.

10. An antibody which selectively binds to a protein of claim 8.

11. A method for producing a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions;

the method comprising the step of culturing the host cell of claim 4 under conditions in which the nucleic acid molecule is expressed.

12. A method for detecting the presence of a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions;

in a sample, the method comprising the steps of:

i) contacting the sample with a compound which selectively binds to the protein; and

ii) determining whether the compound binds to the protein in the sample.

13. The method of claim 12, wherein the compound which binds to the protein is an antibody.

14. A kit comprising reagents used for the method of claim 12, wherein the reagents comprise a compound which selectively binds to a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a peptide comprising at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions.

15. A method for detecting the presence of a nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule which encodes a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a nucleic acid molecule which encodes a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a nucleic acid molecule which encodes a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions;

in a sample, the method comprising the steps of:

i) contacting the sample with a nucleic acid probe or primer which selectively hybridizes to the nucleic acid molecule; and

ii) determining whether the nucleic acid probe or primer binds to a nucleic acid molecule in the sample.

16. The method of claim 15, wherein the sample comprises mRNA molecules and is contacted with a nucleic acid probe.

17. A kit comprising reagents used for the method of claim 15, wherein the reagents comprise a compound which selectively hybridizes to a nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule which encodes a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a nucleic acid molecule which encodes a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a nucleic acid molecule which encodes a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions.

18. A method for identifying a compound which binds to a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2;

b) a fragment of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:2; and

c) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions,

the method comprising the steps of:

i) contacting the protein, or a cell expressing the protein with a test compound; and

ii) determining whether the protein binds to the test compound.

19. The method of claim 18, wherein the binding of the test compound to the protein is detected by a method selected from the group consisting of:

a) detection of binding by direct detecting of test compound/protein binding;

b) detection of binding using a competition binding assay;

c) detection of binding using an assay for NT2LP activity.

20. A method for modulating the activity of a protein selected from the group consisting of:

a) a protein comprising the amino acid sequence of SEQ ID NO:2; and

b) a naturally occurring allelic variant of a protein comprising the amino acid sequence of SEQ ID NO:2, wherein the protein is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 under stringent conditions,

the method comprising the steps of:

i) contacting a cell expressing the protein with a compound which binds to the protein in a sufficient concentration to modulate the activity of the protein.

TGTGGAATGTGGAGAACCAAGCACGGAGGACTGCAGCCTGCCCGCCCTTC
ACCAGGGTCTGCGAGCTTTACCGACGGGAATCGCTGCGTGGACGCGGCCG
AGGCGTGTACAGCAGACGAGCGGTGCCAGCAGCTGCGCTCTGAGTACGTG
GCACGATGCCTGGGCCGGGCAGCGCCCGGGGGCAGGCCGGGACCCGGGGG
CTGCGTGCGCTCCCGCTGCCGCCGACCCCTGCGCCGCTTCTTCGCGCGTG
GGCCTCCGGCGCTCACGCATGCGCTGCTCTTCTGCGGCTGCGAAGGCTCC
GCGTGCGCCGAGCGCCGGCGCCAGACTTTCGCGCCCGCCTGCGCGTTCTC
CGGCCCGGGGTTGGTGCCGCCCTCTTGCCTGGAGCCCCTGGAGCGCTGCG
AGCGCAGCCGCCTGTGCCGGCCCCGTCTCCTTGCCTTCCAGGCCTCATGC
GCTCCCGCGCCCGGCTCCCGCGACCGCTGCCCGGAGGAGGGGGGCCCGCG
TTGTCTGCGCGTCTACGCAGGCCTCATAGGCACCGTGGTCACCCCCAACT
ACCTGGACAACGTGAGCGCGCGCGTTGCGCCCTGGTGCGGCTGTGCGGCC
ANGTGGAAACCGGCGCGAAGAATGCGAAGCCTTCCGCAAGCTCTTTACAA
NGGAACCCCTGCTTGGGTGAGGGGGCCTGGAGGTCCCGGGGAACCACGGA
TGTCTGTGGCCCAATCCAAGCTGCCTGGCCCGTGGGTCTTATTTACGTCG
CATCATGTTTGGTGTGGGCGATGGACAGTGTGCACATGCCATGATGGTGC
CATACAAGCCTTTGACAGCTTGCAGCCATCAGTTCTGCAGGACCAGACTG
CTGGGTGCTGTTTCCCGCGGGCAAGGCACGAGTGGCCTGAGAAGAGCTGG
AGGCAGAAACAGTCCTTGTTTTGTCCTAACGCCCAAGGTGTCCTGGCTGT
ATGCACTCACTGCCCTGGCTCTCCAGGCCCTGCTCTGATTAGGAACATGA
ACCGTGGACGACACAGCTG

CGMWRTKHGGLQPARPSPGSASFTDGNRCVDAAEACTADERCQQLRSEYVARCLGRAAPG
GRPGPGGCVRSRCRRPLRRFFARGPPALTHALLFCGCEGSACAERRRQTFAPACAFSGPG
LVPPSCLEPLERCERSRLCRPRLLAFQASCAPAPGSRDRCPEEGGPRCLRVYAGLIGTVV
TPNYLDNVSARVAPWCGCAAXWKPARRMRSLPQALYXGTPAWVRGPGGPGEPRMSVAQSK
LPGPWVLFTSHHVWCGRWTVCTCHDGAIQAFDSLQPSVLQDQTAGCCFPRARHEWPEKSW
RQKQSLFCPNAQGVLAVCTHCPGSPGPALIRNMNRGRHSX

Fig. 1

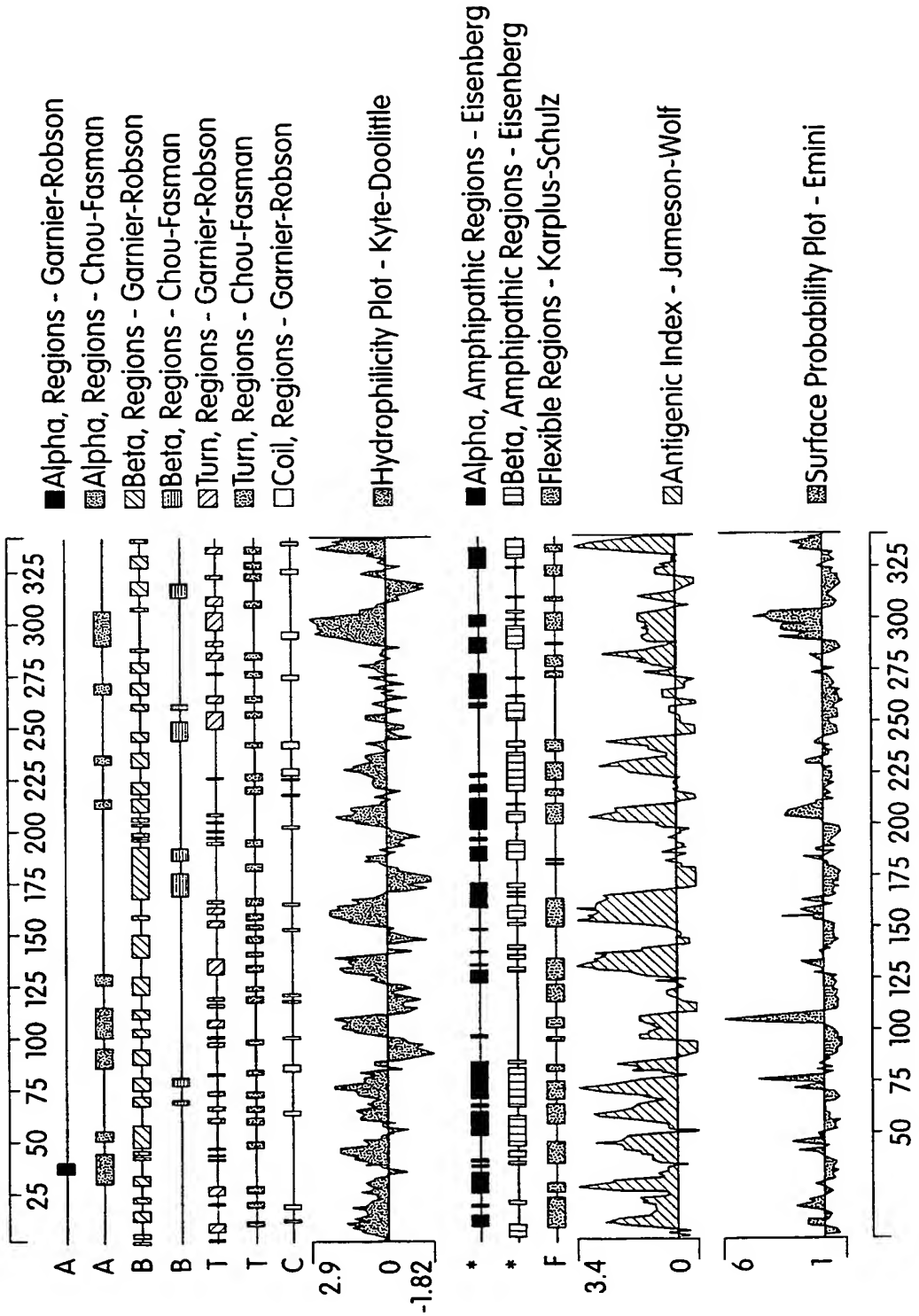

Fig. 2

```
O08842NRTRmouse          .......... .MILANAFCL FFFLDETLRS LASPSSPQGS ELHGWRPQVD
P97785GDNFRamouse        .......... .MFLATLYFV LPLLDLLMSA EVS.....G. ...GDR..LD
AF045162chickGDNF4       .......... .MRGILYFCT LILLEGMAEA VSS..... ....SR...D
AF020305GDNFRa3mouse MGLSWSPRPP LLMILLLVLS LWLPLGAGNS LAT..... ...ENRFVNS
gdnfrorfaa               .......... .......... .......... .......... ..........

O08842NRTRmouse          CVRANELCAA ESNCSSRYRT LRQCLAGRDR NTMLAN.... .KECQAALEV
P97785GDNFRamouse        CVKASDQCLK EQSCSTKYRT LRQCVAGKET NFSLTSGLEA KDECRSAMEA
AF045162chickGDNF4       CLQAGESCTN DPICSSKFRT LRQCIAGNGA NKLGPD...A KNQCRSTVTA
AF020305GDNFRa3mouse CTQARKKCEA NPACKAAYQH LGSCTSSLSR PLPLEES.AM SADCLEAAEQ
gdnfrorfaa               .......... .......... ......... ........... ..........

O08842NRTRmouse          LQESPLYDCR CKRGMKKELQ CLQIYWSIHL GLTEGEEFYE ASPYEPVTSR
P97785GDNFRamouse        LKQKSLYNCR CKRGMKKEKN CLRIYWSMYQ SLQ.GNDLLE DSPYEPVNSR
AF045162chickGDNF4       LLSSQLYGCK CKRGMKKEKH CLSVYWSIHH TLMEGMNVLE SSPYEPF.IR
AF020305GDNFRa3mouse LRNSSLIDCR CHRRMKHQAT CLDIYWTVHP ARSLGDYELD VSPYEDT.VT
gdnfrorfaa               .......... .......... CG.MWRTKHG GLQ..... ..PARPS...

O08842NRTRmouse          LSDIFRLASI FSGTGADPVV SAKSNHCLDA AKACNLNDNC KKLRSSYISI
P97785GDNFRamouse        LSDIFRAVPF ISDVFQQVEH ISKGNNCLDA AKACNLDDTC KKYRSAYITP
AF045162chickGDNF4       GFDYVRLASI TAGSENE... VTQVNRCLDA AKACNVDEMC QRLRTEYVSF
AF020305GDNFRa3mouse SKPWKMNLSK LNMLKPD... ...SDLCLKF AMLCTLHDKC DRLRKAYGEA
gdnfrorfaa               ......PGSA SFTD..... ...GNRCVDA AEACTADERC QQLRSEYVAR

O08842NRTRmouse          CNREISP... ..TERCNRRK CHKALRQFFD RVPSEYTYRM LFCSC..QDQ
P97785GDNFRamouse        CTTSMS.... ..NEVCNRRK CHKALRQFFD KVPAKHSYGM LFCSC..RDV
AF045162chickGDNF4       CIRRLAR... ..ADTCNRSK CHKALRKFFD RVPPEYTHEL LFCPC..EDT
AF020305GDNFRa3mouse CS..... ..GIRCQRHL CLAQLRSFFE KAAESHAQGL LLCPCPPEDA
gdnfrorfaa               CLGRAAPGGR PGPGGCVRSR CRRPLRRFFA RGPPALTHAL LFCGC..EGS

O08842NRTRmouse          ACAERRRQTI LPSCSYEDK. .EKPNCLDLR SLCRTDHLCR SRLADFHANC
P97785GDNFRamouse        ACTERRRQTI VPVCSYEER. .ERPNCLNLQ DSCKTNYICR SRLADFFTNC
AF045162chickGDNF4       ACAERRRQTI VPACSYESK. .EKPNCLAPL DSCRENYVCR SRYAEFQFNC
AF020305GDNFRa3mouse GCGERRRNTI APSCALPS.. .VTPNCLDLR SFCRADPLCR SRLMDFQTHC
gdnfrorfaa               ACAERRRQTF APACAFSGPG LVPPSCLEPL ERCERSRLCR PRLLAFQASC

O08842NRTRmouse          RASYRTITSC PADNYQACLG SYAGMIGFDM TPNYVDSNPT GIVVSPWCNC
P97785GDNFRamouse        QPESRSVSNC LKENYADCLL AYSGLIGTVM TPNYIDS..S SLSVAPWCDC
AF045162chickGDNF4       QPSLQTASGC RRDSYAACLL AYTGIIGSPI TPNYIDN..S TSSIAPWCTC
AF020305GDNFRa3mouse HP.MDILGTC ATE.QSRCLR AYLGLIGTAM TPNFISK..V NTTVALSCTC
gdnfrorfaa               APAPGSRDRC PEEGGPRCLR VYAGLIGTVV TPNYLDN..V SARVAPWCGC
```

Fig. 3

```
O08842NRTRmouse          RGSGNMEEEC EKFLKDFTEN PCLRNAIQAF GNGTDVNMSP KGP.....TF
P97785GDNFRamouse        SNSGNDLEDC LKFLNFFKDN TCLKNAIQAF GNGSDVTMWQ PAPPVQTTTA
AF045162chickGDNF4       NASGNRQEEC ESFLHLFTDN VCLQNAIQAF GNGTYLNAAT APS.....IS
AF020305GDNFRa3mouse     RGSGNLQDEC EQLERSFSQN PCLVEAIAAK MRFHRQLFSQ DWA.....DS
gdnfrorfaa               AAXWKPARRM RSLPQALYXG TPAWVRGPGG PGEPRMSVAQ SKLPG..PWV

O08842NRTRmouse          SATQAPRVEK TPSLPDDLSD STSLGTSVIT TCTSIQEQ.. GLKANNSKEL
P97785GDNFRamouse        MTTTAFRIKN KPLGP..AGS ENEIPTHVLP PCANLQAQ.. KLKSNVSGST
AF045162chickGDNF4       PTTQMYKQER NANRAA.ATL SENIFEHLQP TKVAGEER.. LLRGSTRLSS
AF020305GDNFRa3mouse     TFSVVQQQNS NPALR..LQP RLPILSFSIL PLILLQTL.. W.........
gdnfrorfaa               LFTSHHVWCG RWTVCTCHDG AIQAFDSLQP SVLQDQTAGC CFPRARHEWP

O08842NRTRmouse          SMCFTELTTN ISPGSKKVIK LYSGSCRARL STALTALP.. .......LLM
P97785GDNFRamouse        HLCLSDNDYG KDGLAGASSH ITTKSMAAPP SCGLSSLPVM VFTALAALLS
AF045162chickGDNF4       ...ETSSPAA PCHQAASLLO LWLPPTLAVL SHFMM .... ..........
AF020305GDNFRa3mouse     .......... .......... .......... .......... ..........
gdnfrorfaa               EKSWRQRQSL FCPNAQGVLA VCTHCPGSPG PALIRNMN.. .......RGR

O08842NRTRmouse          VTLA...
P97785GDNFRamouse        VSLAETS
AF045162chickGDNF4       .......
AF020305GDNFRa3mouse     .......
gdnfrorfaa               HSX....
```

Fig. 3 (continued)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MILLENNIUM PHARMACEUTICALS, INC.
(B) STREET: 640 MEMORIAL DRIVE
(C) CITY: CAMBRIDGE
(D) STATE: MASSACHUSETTS
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 02139
(G) TELEPHONE:
(H) TELEFAX:

(ii) TITLE OF INVENTION: GFRα-X, A NOVEL GLIAL-DERIVED NEUROTROPHIC FACTOR RECEPTOR AND USES THEREFOR

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LAHIVE & COCKFIELD, LLP
(B) STREET: 28 STATE STREET
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: US
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US99/
(B) FILING DATE: 25 MARCH 1999
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/080,070
(B) FILING DATE: 31 MARCH 1998

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MANDRAGOURAS, AMY E.
(B) REGISTRATION NUMBER: 36,207
(C) REFERENCE/DOCKET NUMBER: MNI-021PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617)227-7400
(B) TELEFAX: (617)742-4214

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1019 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1017

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TGT GGA ATG TGG AGA ACC AAG CAC GGA GGA CTG CAG CCT GCC CGC CCT      48
Cys Gly Met Trp Arg Thr Lys His Gly Gly Leu Gln Pro Ala Arg Pro
 1               5                  10                  15

TCA CCA GGG TCT GCG AGC TTT ACC GAC GGG AAT CGC TGC GTG GAC GCG      6
Ser Pro Gly Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala
            20                  25                  30

GCC GAG GCG TGT ACA GCA GAC GAG CGG TGC CAG CAG CTG CGC TCT GAG     144
Ala Glu Ala Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu
        35                  40                  45

TAC GTG GCA CGA TGC CTG GGC CGG GCA GCG CCC GGG GGC AGG CCG GGA     192
Tyr Val Ala Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly
    50                  55                  60

CCC GGG GGC TGC GTG CGC TCC CGC TGC CGC CGA CCC CTG CGC CGC TTC     240
Pro Gly Gly Cys Val Arg Ser Arg Cys Arg Arg Pro Leu Arg Arg Phe
65                  70                  75                  80

TTC GCG CGT GGG CCT CCG GCG CTC ACG CAT GCG CTG CTC TTC TGC GGC     288
Phe Ala Arg Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly
                85                  90                  95

TGC GAA GGC TCC GCG TGC GCC GAG CGC CGG CGC CAG ACT TTC GCG CCC     336
Cys Glu Gly Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro
            100                 105                 110

GCC TGC GCG TTC TCC GGC CCG GGG TTG GTG CCG CCC TCT TGC CTG GAG     384
Ala Cys Ala Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu
        115                 120                 125

CCC CTG GAG CGC TGC GAG CGC AGC CGC CTG TGC CGG CCC CGT CTC CTT     432
Pro Leu Glu Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu
    130                 135                 140
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GCC TTC CAG GCC TCA TGC GCT CCC GCG CCC GGC TCC CGC GAC CGC TGC     480
Ala Phe Gln Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys
145                 150                 155                 160

CCG GAG GAG GGG GGC CCG CGT TGT CTG CGC GTC TAC GCA GGC CTC ATA     528
Pro Glu Glu Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile
                165                 170                 175

GGC ACC GTG GTC ACC CCC AAC TAC CTG GAC AAC GTG AGC GCG CGC GTT     576
Gly Thr Val Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val
            180                 185                 190

GCG CCC TGG TGC GGC TGT GCG GCC ANG TGG AAA CCG GCG CGA AGA ATG     624
Ala Pro Trp Cys Gly Cys Ala Ala Xaa Trp Lys Pro Ala Arg Arg Met
        195                 200                 205

CGA AGC CTT CCG CAA GCT CTT TAC AAN GGA ACC CCT GCT TGG GTG AGG     672
Arg Ser Leu Pro Gln Ala Leu Tyr Xaa Gly Thr Pro Ala Trp Val Arg
    210                 215                 220

GGG CCT GGA GGT CCC GGG GAA CCA CGG ATG TCT GTG GCC CAA TCC AAG     720
Gly Pro Gly Gly Pro Gly Glu Pro Arg Met Ser Val Ala Gln Ser Lys
225                 230                 235                 240

CTG CCT GGC CCG TGG GTC TTA TTT ACG TCG CAT CAT GTT TGG TGT GGG     768
Leu Pro Gly Pro Trp Val Leu Phe Thr Ser His His Val Trp Cys Gly
                245                 250                 255

CGA TGG ACA GTG TGC ACA TGC CAT GAT GGT GCC ATA CAA GCC TTT GAC     816
Arg Trp Thr Val Cys Thr Cys His Asp Gly Ala Ile Gln Ala Phe Asp
            260                 265                 270

AGC TTG CAG CCA TCA GTT CTG CAG GAC CAG ACT GCT GGG TGC TGT TTC     864
Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys Cys Phe
        275                 280                 285

CCG CGG GCA AGG CAC GAG TGG CCT GAG AAG AGC TGG AGG CAG AAA CAG     912
Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln
    290                 295                 300

TCC TTG TTT TGT CCT AAC GCC CAA GGT GTC CTG GCT GTA TGC ACT CAC     960
Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His
305                 310                 315                 320

TGC CCT GGC TCT CCA GGC CCT GCT CTG ATT AGG AAC ATG AAC CGT GGA    1008
Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly
                325                 330                 335

CGA CAC AGC TG                                                     1019
Arg His Ser
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 339 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Cys Gly Met Trp Arg Thr Lys His Gly Gly Leu Gln Pro Ala Arg Pro
 1               5                  10                  15

Ser Pro Gly Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala
            20                  25                  30

Ala Glu Ala Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu
        35                  40                  45

Tyr Val Ala Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly
    50                  55                  60

Pro Gly Gly Cys Val Arg Ser Arg Cys Arg Arg Pro Leu Arg Arg Phe
65                  70                  75                  80

Phe Ala Arg Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly
                85                  90                  95

Cys Glu Gly Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro
            100                 105                 110

Ala Cys Ala Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu
        115                 120                 125

Pro Leu Glu Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu
    130                 135                 140

Ala Phe Gln Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys
145                 150                 155                 160

Pro Glu Glu Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile
                165                 170                 175

Gly Thr Val Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val
            180                 185                 190

Ala Pro Trp Cys Gly Cys Ala Ala Xaa Trp Lys Pro Ala Arg Arg Met
        195                 200                 205

Arg Ser Leu Pro Gln Ala Leu Tyr Xaa Gly Thr Pro Ala Trp Val Arg
    210                 215                 220

Gly Pro Gly Gly Pro Gly Glu Pro Arg Met Ser Val Ala Gln Ser Lys
225                 230                 235                 240
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Leu Pro Gly Pro Trp Val Leu Phe Thr Ser His His Val Trp Cys Gly
                245                 250                 255

Arg Trp Thr Val Cys Thr Cys His Asp Gly Ala Ile Gln Ala Phe Asp
            260                 265                 270

Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys Cys Phe
        275                 280                 285

Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln
    290                 295                 300

Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His
305                 310                 315                 320

Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly
                325                 330                 335

Arg His Ser
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1017 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1017

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TGT GGA ATG TGG AGA ACC AAG CAC GGA GGA CTG CAG CCT GCC CGC CCT      48
Cys Gly Met Trp Arg Thr Lys His Gly Gly Leu Gln Pro Ala Arg Pro
 1               5                  10                  15

TCA CCA GGG TCT GCG AGC TTT ACC GAC GGG AAT CGC TGC GTG GAC GCG      96
Ser Pro Gly Ser Ala Ser Phe Thr Asp Gly Asn Arg Cys Val Asp Ala
            20                  25                  30

GCC GAG GCG TGT ACA GCA GAC GAG CGG TGC CAG CAG CTG CGC TCT GAG     144
Ala Glu Ala Cys Thr Ala Asp Glu Arg Cys Gln Gln Leu Arg Ser Glu
        35                  40                  45

TAC GTG GCA CGA TGC CTG GGC CGG GCA GCG CCC GGG GGC AGG CCG GGA     192
Tyr Val Ala Arg Cys Leu Gly Arg Ala Ala Pro Gly Gly Arg Pro Gly
    50                  55                  60
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CCC GGG GGC TGC GTG CGC TCC CGC TGC CGC CGA CCC CTG CGC CGC TTC     240
Pro Gly Gly Cys Val Arg Ser Arg Cys Arg Arg Pro Leu Arg Arg Phe
 65                  70                  75                  80

TTC GCG CGT GGG CCT CCG GCG CTC ACG CAT GCG CTG CTC TTC TGC GGC     288
Phe Ala Arg Gly Pro Pro Ala Leu Thr His Ala Leu Leu Phe Cys Gly
                 85                  90                  95

TGC GAA GGC TCC GCG TGC GCC GAG CGC CGG CGC CAG ACT TTC GCG CCC     336
Cys Glu Gly Ser Ala Cys Ala Glu Arg Arg Arg Gln Thr Phe Ala Pro
            100                 105                 110

GCC TGC GCG TTC TCC GGC CCG GGG TTG GTG CCG CCC TCT TGC CTG GAG     384
Ala Cys Ala Phe Ser Gly Pro Gly Leu Val Pro Pro Ser Cys Leu Glu
        115                 120                 125

CCC CTG GAG CGC TGC GAG CGC AGC CGC CTG TGC CGG CCC CGT CTC CTT     432
Pro Leu Glu Arg Cys Glu Arg Ser Arg Leu Cys Arg Pro Arg Leu Leu
    130                 135                 140

GCC TTC CAG GCC TCA TGC GCT CCC GCG CCC GGC TCC CGC GAC CGC TGC     480
Ala Phe Gln Ala Ser Cys Ala Pro Ala Pro Gly Ser Arg Asp Arg Cys
145                 150                 155                 160

CCG GAG GAG GGG GGC CCG CGT TGT CTG CGC GTC TAC GCA GGC CTC ATA     528
Pro Glu Glu Gly Gly Pro Arg Cys Leu Arg Val Tyr Ala Gly Leu Ile
                165                 170                 175

GGC ACC GTG GTC ACC CCC AAC TAC CTG GAC AAC GTG AGC GCG CGC GTT     576
Gly Thr Val Val Thr Pro Asn Tyr Leu Asp Asn Val Ser Ala Arg Val
            180                 185                 190

GCG CCC TGG TGC GGC TGT GCG GCC ANG TGG AAA CCG GCG CGA AGA ATG     624
Ala Pro Trp Cys Gly Cys Ala Ala Xaa Trp Lys Pro Ala Arg Arg Met
        195                 200                 205

CGA AGC CTT CCG CAA GCT CTT TAC AAN GGA ACC CCT GCT TGG GTG AGG     672
Arg Ser Leu Pro Gln Ala Leu Tyr Xaa Gly Thr Pro Ala Trp Val Arg
    210                 215                 220

GGG CCT GGA GGT CCC GGG GAA CCA CGG ATG TCT GTG GCC CAA TCC AAG     720
Gly Pro Gly Gly Pro Gly Glu Pro Arg Met Ser Val Ala Gln Ser Lys
225                 230                 235                 240

CTG CCT GGC CCG TGG GTC TTA TTT ACG TCG CAT CAT GTT TGG TGT GGG     768
Leu Pro Gly Pro Trp Val Leu Phe Thr Ser His His Val Trp Cys Gly
                245                 250                 255

CGA TGG ACA GTG TGC ACA TGC CAT GAT GGT GCC ATA CAA GCC TTT GAC     816
Arg Trp Thr Val Cys Thr Cys His Asp Gly Ala Ile Gln Ala Phe Asp
            260                 265                 270
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AGC TTG CAG CCA TCA GTT CTG CAG GAC CAG ACT GCT GGG TGC TGT TTC     864
Ser Leu Gln Pro Ser Val Leu Gln Asp Gln Thr Ala Gly Cys Cys Phe
        275                 280                 285

CCG CGG GCA AGG CAC GAG TGG CCT GAG AAG AGC TGG AGG CAG AAA CAG     912
Pro Arg Ala Arg His Glu Trp Pro Glu Lys Ser Trp Arg Gln Lys Gln
    290                 295                 300

TCC TTG TTT TGT CCT AAC GCC CAA GGT GTC CTG GCT GTA TGC ACT CAC     960
Ser Leu Phe Cys Pro Asn Ala Gln Gly Val Leu Ala Val Cys Thr His
305                 310                 315                 320

TGC CCT GGC TCT CCA GGC CCT GCT CTG ATT AGG AAC ATG AAC CGT GGA    1008
Cys Pro Gly Ser Pro Gly Pro Ala Leu Ile Arg Asn Met Asn Arg Gly
                325                 330                 335

CGA CAC AGC                                                        1017
Arg His Ser
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06631

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/705; C12N 15/12, 15/63, 15/74, 15/79; C07H 21/00
US CL :530/350; 536/23.5, 24.31; 435/69.1, 252.3, 254.11, 320.1, 325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.5, 24.31; 435/69.1, 252.3, 254.11, 320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Medline, WPI
search terms: Glial Derived Neurotrophic Factor Receptor, GDNF Receptor, DNA, cloning

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JING et al. GDNF-Induced Activation of the Ret Protein Tyrosine Kine is mediated by GDNFR-α, a Novel Receptor for GDNF. Cell. 28 June 1996, Vol. 85, pages 1113-1124.	1-9, 11, 14, 17
A	JING et al. GFRα-2 and GFRα-3 are Two New Receptors for Ligands of the GDNF Family. The Journal of Biological Chemistry. 26 December 1997, Vol. 272, pages 33111-33117.	1-9, 11, 14, 17
A	BALOH et al. TrnR2, a Novel Receptor that Mediates Neurturin and GDNF Signaling through Ret. Neuron, May 1997, Vol. 18, pages 793-802.	1-9, 11, 14, 17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
16 JUNE 1999

Date of mailing of the international search report
16 JUL 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06631

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NOMOTO et al. Molecular Cloning and Expression Analysis of GFRα-3, a Novel cDNA Related to GDNFRα and NTNRα. Biochemical and Biophysical Research Communications. 27 March 1998, Vol. 244, pages 849-853.	1-9, 11, 14, 17
X, P ----- Y, P	EMBL/GenBank Database, Accession No. AU035938, SASAKI, Z., 'Construction of Mouse Full Length-Enriched cDNA Libraries,' abstract, Katsuyuki Hashimoto, National Institute of Infectious Diseases, Division of Genetic Resources, October 1998, see whole document.	1 ---------- 2-9, 11, 14, 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06631

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9, 11, 14, and 17

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-9, 11, 14, and 17, drawn to nucleic acid and the encoded polypeptide having SEQ ID NO: 2.
Group II, claim 10, drawn to antibodies.
Group III, claims 12 and 13, drawn to a method of detecting the presence of a polypeptide having SEQ ID NO: 2.
Group IV, claims 15 and 16, drawn to a method of detecting the presence of the nucleic acid molecule encoding SEQ ID NO: 2.
Group V, claims 18 and 19, drawn to a method of identifying a compound that binds a protein having SEQ ID NO: 2.
Group VI, claim 20, drawn to a method of modulating the activity of a protein having SEQ ID NO: 2.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of Group I is the nucleic acid sequence encoding the polypeptide having SEQ ID NO: 2. The special technical feature of Group II is the antibodies that bind to the polypeptide but does not have the amino acid sequence of the polypeptide. The special technical feature of Group III is a method of detecting the presence of a polypeptide using a compound that bind to the polypeptide. The special technical feature of Group IV is a method of detecting the presence of a nucleic acid molecule using a nucleic acid encoding SEQ ID NO: 2. The special technical feature of Group V is a method of identifying a compound that binds a protein having SEQ ID NO: 2 using the polypeptide and a test compound. The special technical feature of Group VI is a method of modulating the activity of a protein using a cell expressing the protein and a compound that binds the protein. The special technical feature of each group is not the same or does not correspond to the special technical feature of any other group because the products of Groups I and II are structurally and functionally distinct and the methods of Groups III-VI require different method steps and starting reagents for achieving different goals. The groups are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.